A Cohort Study of Systemic Markers of Inflammation and Oxidative Stress and Incidence of Esophageal Adenocarcinoma

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Abstract

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Esophageal adenocarcinoma (EA) incidence has increased dramatically in the Western world over several decades, while survival remains poor. Persons with Barrett's esophagus (BE) experience a higher risk for progression to EA; they are typically followed in long-term surveillance programs involving periodic endoscopy with biopsies so as to identify early-stage cancers. Currently, no medical, surgical or lifestyle interventions have been observed to safely lower EA risk. Recent studies have shown that elevated pre-diagnostic levels of serum inflammation markers may be predictive of cancers of the breast, colon, and lung, but their rolein predicting EA is unknown.

In this dissertation project, we investigated whether elevated markers of inflammation, including C-reactive protein (CRP), interleukin-6 (IL6), and soluble tumor necrosis factor receptors I & II (sTNF-RI & sTNF-RII), and markers of oxidative stress, including F2-isoprostanes, could predict progression to EA in the Seattle Barrett's Esophagus Study (SBES), a prospective cohort of 397 BE patients 45 of whom developed EA. We also assessed the intra-individual variability and reliability of these inflammation markers. Additionally, we evaluated the correlates of telomere length in a subset of 234 persons from the SBES cohort.

We observed that persons with CRP levels above the median value of 1.9 mg/L were at a two-fold increased risk for EA compared to those below (adjusted HR 1.77; 95% CI 0.93-3.37, p-trend for continuous CRP=0.04). Persons with IL6 levels above the median had a two-fold increased risk for EA (age- and gender-adjusted HR 1.95; 95%CI 1.03-3.72) but no significant trend was observed (p-trend = 0.94). No evidence of an association was found between EA risk and elevated levels of sTNF receptors or F2-isoprostanes. Analyses restricted to men revealed slightly stronger associations, but the overall conclusions remained the same.

In a reliability study involving a subset of 360 participants from the SBES, we observed that the reliability over time, evaluated as intra-class correlations (ICCs), was excellent for sTNF receptors ($ICC_{sTNF\text{-}RI}$=0.89, $ICC_{sTNF\text{-}RII}$=0.85) and fair to good for CRP and IL-6 (ICC_{CRP}=0.55, $ICC_{IL\text{-}6}$=0.57). Moreover, the ICCs for CRP & IL-6 were lower among samples stored for over 13 years prior to laboratory analysis compared to samples stored for less than 13 years, but those for sTNF receptors were unaffected by storage time.

In a cross-sectional analysis to assess the correlates of leukocyte telomere length (LTL), age, gender and cigarette pack-years of smoking were significantly associated with LTL. We

observed that elevated sTNF-RI levels were associated with short telomeres (Adjusted OR comparing extreme tertiles = 2.19, 95% CI 1.00-4.85, p-trend for continuous sTNF-RI = 0.02). There were no significant associations observed between short LTL and higher levels of other inflammation markers, including CRP, IL-6, sTNF-RII, and F2-isoprostanes. We also did not find any association between short LTL and obesity or obesity-related biomarkers including leptin, adiponectin, glucose and insulin.

Our findings suggest that systemic inflammation markers, including CRP and possibly IL-6, can predict progression to EA among persons with BE. Continued follow-up of this and other larger cohorts is needed to further understand the relationship between inflammation, telomeres and cancer, and possibly evaluate inflammation markers as tools for clinical risk stratification in persons with BE.

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DEDICATION

This work is dedicated to my incredible family,

Sharadchandra, Sulbha, Swanand,

Nihar & Nishka,

whose love, support and encouragement make it all worthwhile!!

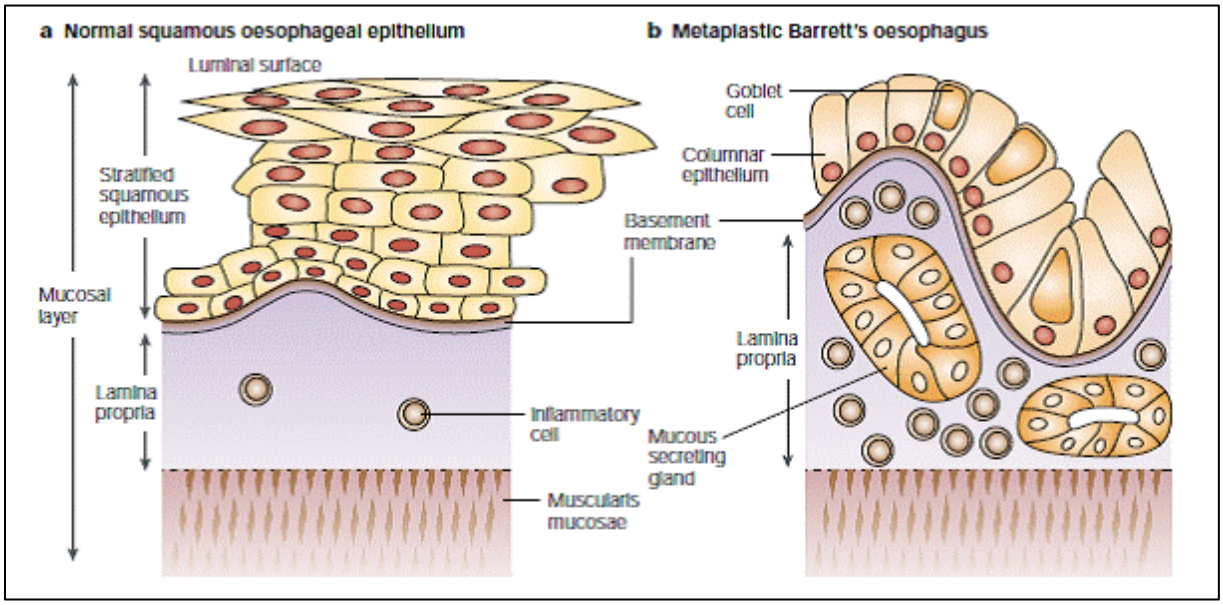
INTRODUCTION

A rapid increase in incidence of and mortality from esophageal adenocarcinoma (EA) has been observed over the past four decades in the Western world.(1-4) Emerging evidence suggests that EA has also become more common in some Asian populations, such as in Singapore and Japan, where the disease has previously been almost unknown.(5, 6) In the US, there has been an approximate 463% increase in incidence among white men and a 335% increase among white women over a 30 year period from 1975-2004.(7) Although the absolute incidence of EA varies dramatically by gender and race, few demographic groups have been spared from the increases. Moreover the survival of persons with EA remains abysmal, with most succumbing to the disease within a year.(8, 9) Increasing incidence coupled with high mortality make this disease an important public health problem.

The only identified precursor of EA is Barrett's esophagus (BE), a condition of the lower end of the esophagus where the normal squamous epithelium is replaced by a metaplastic columnar type of intestinal epithelium (Figure 1).(10) BE develops in approximately 10-15% of patients with gastro-esophageal reflux disease (GERD) and is associated with a 30 fold or higher increase in the incidence of EA.(10, 11) Currently, the only way to identify BE patients that progress to cancer is through periodic endoscopic surveillance coupled with serial biopsies of the Barrett's segment in the hope of identifying early-stage esophageal adenocarcinomas that are more amenable to surgical cure. Although such surveillance is expensive and time-consuming, persons with EA diagnosed through surveillance generally have substantially increased survival as compared to those diagnosed outside of such programs.(12) While persons with BE are at a higher risk of EA development as compared to persons in the general population, their absolute risk of an individual with BE progressing to EA is quite low, approximately 0.4-0.7% per

year.(13, 14) It is not yet clear why the esophageal epithelium in only some persons with BE undergoes a neoplastic change and progresses to cancer, while in most others it remains in a relatively benign condition throughout their life.

Figure 1: Barrett's esophagus: Replacement of the normal stratified squamous epithelium (a) by an intestinal type of columnar epithelium (b)

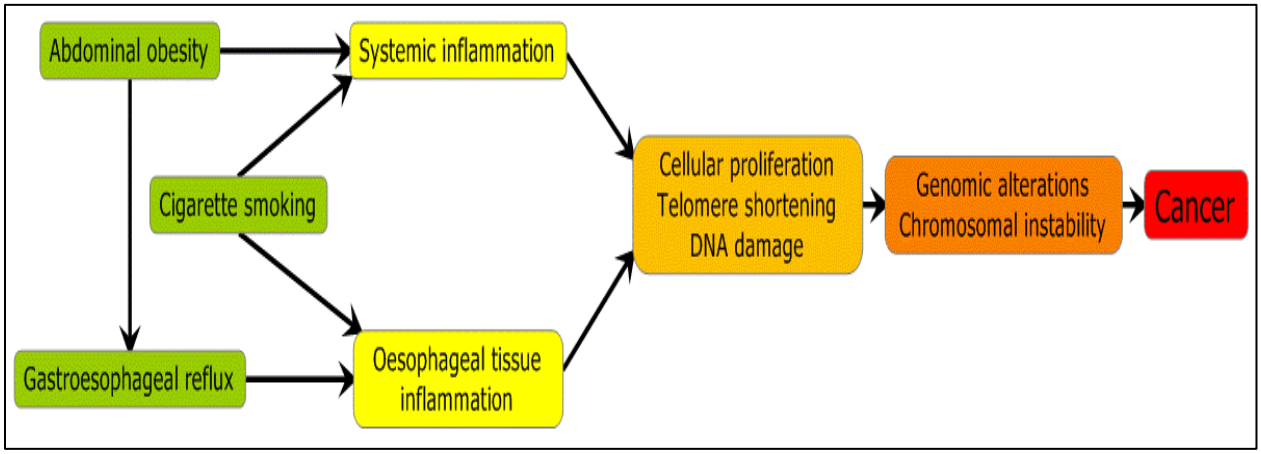


Wild *et al*. Nat. Rev, 2003

Extensive research has identified the likely primary causes for EAs implicating gastroesophageal reflux, obesity (particularly central adiposity), cigarette smoking and, to a lesser extent, diet as potentially modifiable etiologic factors.(15-17) Additionally, a growing number of observational studies suggest that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) may be effective in reducing risk of EA.(18, 19) Decreased risk of progression has also been shown among consumers of anti-oxidant vitamins such as vitamin C & vitamin E.(17) Other non-modifiable clinical and demographic factors that have shown promise in being predictive of neoplastic transformation in Barrett's patients are male gender(20), older age(20), and length of Barrett's segment.(21)

One common aspect among all of the above mentioned modifiable risk factors for EA is their established link with inflammation and oxidative damage. Abdominal obesity is generally considered to be a low-grade inflammatory state such that inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF- α), and acute phase proteins like C-reactive protein (CRP) have been observed to be systematically elevated in obese individuals.(22, 23) GERD can expose the lower esophagus to acid and bile salts which may be toxic to the epithelium and may cause inflammation of the esophageal tissue.(24) Similarly, it is well established that tobacco smoke contains thousands of chemicals that are known to cause inflammation and oxidative damage to cells.(25) NSAID's and anti-oxidant vitamins also clearly interrupt the inflammation pathway. The observation that these factors are also etiologically related to development of EA points to a possible role of inflammation in neoplastic transformation among Barrett's patients.

Figure 2: Biological pathways connecting inflammation and esophageal adenocarcinoma



Reid *et al*. Nat Can Rev, 2010

Although cancers of the colon, lung and breast have been previously shown to be associated with inflammation markers such as CRP and IL-6(26-33), no epidemiological study

has as yet investigated the central role that chronic inflammation seems to play in the development of adenocarcinoma among BE patients. Biological mechanisms connecting inflammation with EA are depicted in Figure 2. Briefly, a chronic state of systemic and localized inflammation and oxidative stress resulting from GERD, abdominal obesity and/or smoking may promote DNA damage, cellular proliferation and telomere shortening, which in turn can increase the risk of developing clones containing small- and large-scale genomic alterations, eventually leading to widespread chromosomal instability and development of cancer in the Barrett's segment.(15)

In this study, we have sought to understand the as yet untested relationship between inflammation, oxidative stress and EA (Chapter 1). We have investigated whether biomarkers of inflammation, including CRP, IL-6, soluble tumor necrosis factor (sTNF) receptors I and II, and markers of oxidative stress, including F_2-isoprostanes, could predict progression to EA in the Seattle Barrett's esophagus Study (SBES), a cohort of BE patients followed prospectively for development of EA.

Typically, such epidemiological studies involving prospectively collected biospecimens may only have a single blood sample available to them from each participant due to limited number of samples collected. Moreover, samples may be stored for a prolonged time before laboratory analysis, which may in turn cause degradation and affect biomarker measurement. Additionally, as inflammation markers vary in both acute (e.g. infection, injury) as well as chronic inflammatory conditions (e.g. obesity, diabetes, cardiovascular disease, cancer), it is important to determine whether circulating marker levels at a single time point are reflective of an individual's average level over time, relative to other individuals. The existing studies investigating this question are limited either by small sample sizes or short duration (weeks to

months) over which the variability is evaluated.(34-37) None of them have evaluated the effect of long-term storage of blood samples on their reliability.

Understanding long-term within-person variations in inflammation markers is essential to the implementation and interpretation of epidemiologic research on the associations between these biomarkers and health outcomes. Here, we assessed within- and between-person variation in plasma concentrations of CRP, IL-6 and sTNF receptors I & II in a subsample of the SBES cohort, in addition to evaluating the effects of long-term storage of samples (Chapter 2).

Another marker that has been hypothesized to capture a person's long-term history of inflammation and oxidative damage is telomere length in leukocytes of people with Barrett's esophagus. This hypothesis is strengthened by the observation that factors such as insulin resistance, obesity and smoking, that may in turn cause low-grade inflammation and oxidative stress, have been shown to reduce leukocyte telomere length.(38, 39) In a previous analysis in the SBES cohort, shorter leukocyte telomere length was associated with increased risk of progression to EA (adjusted hazard ratio comparing extreme quartiles: 3.45; 95% CI = 1.35–8.78).(40) Later, a case-control study was able to replicate these observations and also reported an association between EA and telomere length.(41) These observations taken together raise the possibility that telomere length may be a correlate of the underlying level of inflammation in persons with BE (Figure 2). Although leukocyte telomere length has been shown to be correlated with inflammation in other populations, no epidemiologic study has evaluated a similar correlation among BE patients.(42, 43)

In Chapter 3, we investigated the correlates of telomere length among a subset of the SBES cohort. In addition to exploring the relationships between leukocyte telomere length and known correlates such as age, gender and lifestyle factors such as smoking and obesity, we also

evaluated whether elevated systemic markers of inflammation and obesity-related markers were associated with short telomere length.

Understanding the underlying mechanisms responsible for progression to adenocarcinoma among persons with Barrett's esophagus is crucial in identifying prevention strategies. If less invasive techniques could assess risk of onset of EA in BE patients, surveillance and prevention efforts could then be targeted to the subset of patients with the highest risk; substantially reducing cost, patient anxiety, and potential morbidity. Inflammatory pathway is one such mechanism that may possibly explain, at least partially, the progression from BE to EA and has potential for modification through interventions. Further study of this pathway is essential to better understand the complex relationship between inflammation and development of EA among persons with Barrett's esophagus.

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CHAPTER 1: Association of plasma markers of inflammation and oxidative stress and esophageal adenocarcinoma risk in a Barrett's esophagus cohort

ABSTRACT

BACKGROUND: Persons with Barrett's esophagus (BE) experience an increased risk of esophageal adenocarcinoma (EA). Recent studies have shown that prediagnostic inflammation marker levels may be predictive of cancers of the breast, colon, and lung, but their role in predicting EA is unknown.

METHODS: We investigated whether biomarkers of inflammation and oxidative stress including C-reactive protein (CRP), Interleukin-6 (IL-6), soluble tumor necrosis factor (sTNF) receptors I and II, and F_2-isoprostanes could predict progression to EA in a prospective cohort of 397 BE patients, 45 of whom developed EA. Participants underwent periodic endoscopy with multiple biopsies, personal interview and blood draw. Most biomarkers were measured in stored plasma samples at two separate times during follow-up, the mean of which served as the primary predictor. Adjusted Hazard Ratios (HR) were estimated using Cox proportional hazards regression.

RESULTS: Persons with CRP levels above the median value of 1.9 mg/L were at a two-fold increased risk for EA compared to those below the median (age- and gender-adjusted HR 1.98; 95% CI 1.05-3.73, p-trend based on continuous measure = 0.01). Further adjustment for obesity and smoking changed results only minimally. Persons with IL6 levels above the median had a two-fold increased risk (age- and gender-adjusted HR 1.95; 95%CI 1.03-3.72) but no evidence of a trend was observed. Increased levels of TNF receptors and isoprostanes were not associated with EA risk.

CONCLUSIONS: These results support the hypotheses that a) inflammation is significantly associated with EA development among BE patients and b) inflammation markers such as CRP and IL6 may be useful in identifying persons at higher EA risk.

Continued follow-up of this and other larger cohorts is needed to further evaluate inflammation markers as tools for clinical risk stratification among BE patients.

INTRODUCTION

Chronic inflammation has been hypothesized to play an important role in the pathogenesis of cancers of the lung, colon, breast, bladder, pancreas and several other organs (1-5). Similarly, oxidative stress has been implicated in cancers of the lung(6), breast(7), prostate(8) and colon(9). Inflammation may contribute to cancer development through a number of mechanisms, including DNA damage, angiogenesis, promotion of cellular proliferation, and inhibition of apoptosis.(1) Inflammatory processes also lead to generation of reactive oxygen species (ROS) which are primarily responsible for the oxidative damage associated with inflammation. These ROS may cause inactivating mutations in tumor suppressor genes or post-translational modifications in DNA repair proteins, thus promoting carcinogenesis.(1, 5)

In the gastrointestinal tract, several diseases involving chronic inflammatory processes have been clearly demonstrated to be associated with cancer: idiopathic inflammatory bowel diseases such as ulcerative colitis and Crohn's disease with colorectal cancer(10), chronic pancreatitis with pancreatic cancer(11), hepatitis B & C with liver cancer(12), and chronic *Helicobacter pylori* gastritis with gastric cancer.(13) Similarly, inflammatory conditions of the esophagus, namely reflux esophagitis and Barrett's esophagus (BE), are strongly implicated in the development of esophageal adenocarcinoma (EA), with BE widely considered to be a pre-malignant lesion for this cancer.(14-16) The inflammatory link with EA is further strengthened by the observation that regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin are associated with decreased risk.(17-19)

The incidence of EA has increased dramatically over the past four decades.(20) Although the relative risk of EA is about 30 times higher in individuals with BE

compared to those without,(21) absolute risk of progression is relatively low (0.4-0.6% per year)(22), and it has proven difficult to identify with accuracy which persons with BE are most likely to develop EA, or whether certain lifestyle or behavior modifications or medical interventions might help prevent EA within this higher-risk population. Previous studies examined obesity, cigarette smoking, gastro-esophageal reflux and diet as potential modifiable risk factors for EA.(16, 23) However, the roles of inflammation and oxidative stress and related markers as potentially modifiable risk factors for EA, as well as part of a panel of markers for risk stratification have not been studied. In this report we assess the association between markers of systemic inflammation and oxidative stress and the subsequent risk of progression to EA in a cohort of BE patients.

METHODS

***Study population, follow-up & cancer ascertainment*:** The Seattle Barrett's Esophagus Study (SBES), is a prospective cohort study aimed at understanding the risk factors and mechanisms underlying neoplastic progression to EA among persons with BE, and detecting such cancers at an early stage. Details of the cohort and protocol have been described previously.(23-25) The study involves periodic endoscopic surveillance for all the participants, with multiple biopsies of the Barrett's segment. The current report includes SBES participants with BE and no history of esophageal cancer enrolled between February 1995 and September 2009. During this time frame, 427 participants were enrolled into the SBES cohort, of whom 411 (96.3%) had at least one follow-up visit and thus were eligible for analyses. For such participants, the clinic visit on or after February 1, 1995 was termed the baseline visit; it included an extensive personal interview, anthropometric assessments, endoscopy with biopsy and collection of blood samples. At subsequent follow-up visits, baseline information was updated and a repeat endoscopy with biopsies was performed as described previously.(14, 15, 26) Briefly, four-quadrant biopsies were obtained at 2 cm intervals from the Barrett's segment (1 cm intervals for those with history of high-grade dysplasia) and were fixed, processed and interpreted by a single pathologist blinded to the exposure status of the participants.(14) Individuals were classified as having BE, low-grade dysplasia, high-grade dysplasia or EA based on their most severe histological diagnosis. Participants with high grade dysplasia at their initial endoscopy (80/411 participants; 19.46%) were endoscoped twice more within 4 months in order to detect any occult cancers missed at baseline endoscopy. EA was defined as invasion of neoplastic epithelium beyond the basement membrane of the esophageal mucosa into the surrounding lamina propria, muscularis mucosa or

submucosa.(14) This study was approved by the Institutional Review Boards at the University of Washington and Fred Hutchinson Cancer Research Center. Written informed consent was obtained from all participants.

***Inflammation marker measurements*:** At enrollment and subsequent clinic visits, fasting blood samples were collected by venipuncture from all participants, processed within 2 hours after collection and stored at -80°C until analysis. Plasma levels of CRP, IL-6 and sTNF receptors were measured using samples from the first two available time points (baseline and first follow-up for most participants, mean duration between two samples 1.8 years). F2-isoprostanes were measured at a single time point (earliest available, baseline for most). Intra- and inter-batch coefficients of variation (CV) were calculated using blinded pooled plasma samples included as quality controls within each batch. Intraclass correlation coefficients (ICC) and 95% confidence intervals (CI) were calculated using the two cytokine measurements available on each participant.

CRP concentrations were measured in never-thawed plasma samples with a high-sensitivity assay using immunonephelometry (Dade Behring Inc, Deerfield, IL) as per the manufacturer's instructions [Interbatch CV 2.9%, ICC(95% CI) 54.9% (47.4,62.5)]. The assay detectable limit was 0.2 mg/L; a value of 0.1 mg/L was assigned to all participants with CRP concentrations below the detection limit (< 1% samples). IL-6 was assayed in never-thawed plasma samples using the Quantikine HS human IL-6 Elisa kit (R&D Systems Inc, Minneapolis, MN; HS600B) according to the manufacturer's instructions [Inter-batch CV 4.4%, intra-batch CV 4.1%, ICC(95% CI) 56.4% (49.4,63.4)]. The limit of quantification for this assay was 0.156 pg/ml and none of the samples assayed below

this level. Samples were run in duplicate with a median duplicate CV of 2.7%; samples with CVs greater than 12% were re-run and the repeat measurements were used for analysis. sTNFR-I and sTNFR-II were measured on previously thawed and re-frozen plasma samples using the MILLIPLEX MAP Human Soluble Cytokine Receptor Panel (Millipore, Billerica, MA; HSCR-32K) according to the manufacturer's instructions [sTNFR-I: inter-batch CV 8.9%, intra-batch CV 5.9%, ICC(95% CI) 89.2% (87.2,91.3); sTNFR-II: inter-batch CV 6.1%, intra-batch CV 2.4%, ICC(95% CI) 84.9% (82.1,87.8)]. The limit of quantification of the Millipore assay was 12.2 pg/ml and all the samples were over this limit. Samples were run in duplicate with a median duplicate CV of 4.1% and 3.4% for sTNFRI and sTNFRII, respectively. F_2-isoprostanes were estimated in never-thawed plasma samples using gas chromatography/ negative ion chemical ionization mass spectrometry (GC/NICI-MS) as described previously.(27, 28) GC/MS analysis was conducted using a 6890N Agilent gas chromatograph coupled to a 5973 quadruple mass spectrometer (Santa Clara, CA) in the negative-ion mode. This assay had a precision of ±3% (every 8^{th} sample tested was injected twice into the gas chromatograph to test precision), accuracy of 97% and a detection limit of 20 pg/ml.

***Other covariates*:** All SBES participants underwent a personal interview conducted by trained staff at their baseline evaluation. Detailed information on their medical, family, and medication history (along with current medication use) was collected in addition to information on lifestyle exposures such as tobacco use and alcohol consumption. Anthropometric measurements including height, weight, and waist, hip, thigh, and abdominal circumferences were carried out using an established protocol.(23) Body mass

index (BMI) was calculated as weight in kg divided by height in meters squared, and categorized as normal weight (<25 kg/m2); overweight (>25 and <30 kg/m2); and obese (>30 kg/m2). Waist-hip ratio (WHR) was computed as the ratio of waist and hip circumferences. Cumulative pack-years of smoking were computed using the number of packs smoked per day and the number of years smoked. The number of alcoholic drinks/day was calculated based on the participants' beer, wine and liquor consumption.

***Statistical Analysis*:** We estimated the associations between markers of inflammation and oxidative stress and the risk of EA by calculating hazard ratios (HR) and 95% confidence intervals (CI) using separate Cox proportional hazards regression models for each biomarker. We used the time to development of EA as the underlying time metric; number of person-years for each participant was computed from the date of baseline visit endoscopy to the date of endoscopic cancer diagnosis or last follow-up. For participants with inflammation marker measurements available at two time points, the mean of the two measures was used as the primary predictor. For those with only a single biomarker measure available, that measure alone was used to predict EA risk. If the outcome occurred during the same visit as a biomarker measurement, only the first measurement was used for risk prediction. A priori, we decided to exclude CRP values over 10 mg/L from our analyses due to the possibility that such elevated concentrations may be the result of acute rather than chronic inflammation.(29, 30) Biomarker concentrations were tested for associations with EA risk using three models: i) unadjusted, ii) age- and gender-adjusted, and iii) adjusted for obesity, smoking and NSAID use in addition to age and gender. These specific confounders were selected based on risk factors of EA

(determined a priori). Analyses were conducted on quartiles of the various biomarkers, as well as by the median level i.e. comparing those above and below the median. Analyses were repeated restricted to males. The number of females was too small to yield meaningful results. Tests for trend were based on the likelihood-ratio test associated with addition of the biomarker being evaluated in its continuous form. A two-sided p-value less than 0.05 was considered statistically significant. The assumption of proportional hazards over time was tested by using the log-likelihood test and comparing models with and without the interaction term between the various biomarkers and time in separate models. Pearson's correlation coefficients were used to evaluate the correlations among cohort characteristics and the evaluated biomarkers. All analyses were performed using STATA statistical package, release 12 (StataCorp, College Station, TX).

RESULTS

Participant characteristics for the 411 individuals eligible for current analysis are presented in table 1. The majority of the cohort participants were Caucasian (96.6%) and male (81.3%), with a mean age of 61.2 years. Over thirty-nine percent of participants were obese, 64% were current or former smokers, 81.5 % reported regular alcohol use in their lifetime and 60.6% had regularly taken NSAIDs at some point in their life. The mean WHR for the entire cohort was 0.95 (males 0.96, females 0.87).

The medians and interquartile ranges for the various biomarkers overall and by quartile are presented in table 2. The distributions among males and females were based on sex-specific quartiles for the respective biomarkers. Overall, the distributions of the various biomarkers evaluated in this study are comparable to other studies of older populations and obese individuals.(31-33)

The Pearson's correlation coefficients between the different biomarkers and risk factors for EA are shown in table 3. The correlations between the biomarkers themselves were small to moderate, from 0.12-0.63, and were statistically significant with the exception of IL6 and isoprostanes. Therefore, these biomarkers were evaluated in separate models with respect to their EA risk. Most of the biomarkers were significantly correlated with age, WHR, and cigarette pack-years, suggesting that these factors might confound the biomarker-EA associations.

Table 4 presents the HRs and 95% CIs for developing EA according to biomarker levels. Of the 411 SBES participants eligible for analyses, 14 individuals had less than 5 months of follow-up and 11 of these developed cancer. Due to an *a priori* concern that cancers diagnosed during this early period of intensive search for occult malignancies may have been present at baseline, these 14 individuals were excluded from the primary

statistical analyses. The remaining 397 participants were followed for a median of 6.5 years (33,635 person-months), and 45 of them developed cancer. Plasma samples were not available for three individuals. For analyses involving CRP, we omitted eight participants for whom all available CRP values were over 10 mg/L. Ultimately, analyses were conducted based on 394 participants for IL6 and sTNF receptors, 386 participants for CRP and 377 participants for isoprostanes.

Mean CRP levels above the median value of 1.9 mg/L were associated with a two-fold increased risk for EA compared to those with values below the median, after adjustment for age and gender (HR 1.98; 95% CI 1.05-3.73). Further adjustment for confounding effects of WHR, smoking and NSAIDs attenuated the association somewhat (HR 1.77; 95% CI 0.93-3.37). We observed a significant trend with increasing CRP concentrations both after age and gender adjustment (p_{trend} = 0.01) and after adjustment for additional risk factors (p_{trend} = 0.04). Analyses limited to men revealed slightly stronger associations with CRP.

Participants with average IL6 levels above the median had a two-fold increased risk for EA (HR 1.95; 95%CI 1.03-3.72) but no evidence of a trend was observed (p_{trend} = 0.94). The increase in risk was more pronounced among males, with an almost three-fold increased risk (HR 2.85, 95% CI 1.38-5.92) after adjustment for age and gender. Overall as well as in the subgroup analysis for males, additional adjustment for obesity, smoking and NSAID had little effect. No evidence of an association between sTNF-RI and EA risk was observed. For sTNF-RII, although univariate analyses overall and among males revealed statistically significant associations, adjustment for confounding factors attenuated the association substantially such that it was no longer statistically significant.

Circulating levels of F2-isoprostanes were not associated with increased risk of development of EA in this cohort.

We repeated our main analyses after adding the 14 individuals with less than 5 months of follow-up that we had initially excluded. The results did not differ in important ways from the associations observed in the main statistical analyses (data not shown). In another sensitivity analysis, we excluded the first five months of follow-up time for all the 397 individuals and repeated the statistical analyses, results of which did not differ from those presented in this study (data not shown). Biological samples utilized in this report had been stored for a number of years before laboratory measurement (mean storage time for the earlier of the two samples from an individual was 12 years). To examine whether this might affect the results, we also conducted a sensitivity analysis in which we included only those individuals whose biological samples had been stored for 10 years or less. We found that while the results for CRP were slightly stronger and those for IL6 were weaker as compared to the main statistical analysis, the overall conclusions remained the same (data not shown). As there were no clinically relevant cut-offs suggestive of acute inflammation for elevated IL-6 or sTNF receptor levels (like the cut-off point of CRP > 10 mg/L), we repeated our analyses after excluding top 5 % values for these variables and did not find any significant differences in the results (data not shown).

DISCUSSION

In this prospective study, we observed that elevated pre-diagnostic blood concentrations of CRP and possibly IL6 are associated with subsequent increased risk of EA among persons with BE, particularly among males. Plasma levels of soluble TNF receptors or isoprostanes were not statistically significantly associated with EA risk.

The role of inflammation and resulting oxidative stress in the development of cancer has been the focus of extensive research.(3, 34, 35) More recently, markers of inflammation, particularly CRP and IL-6, have been reported to be associated with all cancers in prospective and nested case-control studies. Allin *et al.* reported multifactorially adjusted hazard ratios of 1.3 (95%CI 1.0-1.6) for risk of all cancers combined associated with CRP levels over 3 mg/L in a prospective Danish cohort of 10,000 individuals observed for 16 years(36), while Trichopoulos *et al.* showed a 1.2-fold (95% CI, 1.10-1.32) increase in risk of all cancers associated with increased levels of CRP in a Greek cohort.(37) In another prospective study of participants from the Health Aging and Body Composition cohort, Il'yasova *et al.* observed an increased risk for all cancers with increases in blood concentrations of CRP, IL-6 and TNF-α.(38) In studies looking at individual cancer sites, the evidence for a possible role of chronic inflammation in cancer development is the strongest for colon cancer.(36, 38-41) Elevated levels of inflammatory markers have also been shown to be associated with cancers of the lung(36-38, 42, 43) and the breast(37, 38, 43). Oxidative stress has been shown to be associated with cancers at several sites such as colon, lung, prostate and breast.(6-9).

Our study adds to the accumulating evidence for a key role of inflammation in EA development. Exposure of the esophageal epithelium to bile salts and acid resulting from

gastroesophageal reflux can cause chronic inflammation of the lower esophagus and result in increased release of pro-inflammatory mediators such as cytokines and reactive oxygen species within the esophageal tissue.(44-46) These mediators may in turn cause DNA damage and also promote progression.(1, 44) Indeed, in support of this hypothesis, Dvorakova *et al* showed in an experimental study that BE tissue secretes significant amounts of IL-6 resulting in an increased expression of STAT3 transcription factor that may lead to apoptosis resistance and neoplastic conversion.(47) In another study, the levels of ROS were higher in biopsy specimens from BE patients as compared to those from controls suggesting that ROS may play a role in the tissue injury associated with BE.(48) In our own cohort, we have previously shown that leukocyte telomere length, a measure of person's long-term inflammation level and oxidative damage(49) was associated with more than a three-fold increased risk of EA (p-trend 0.009).(50) In another study involving our cohort, we have also reported that anti-inflammatory drugs such as NSAIDs reduce the risk of EA even among those with dysplastic changes in their Barrett's segment.(17) Here, we show a link between pre-diagnostic concentrations of inflammation markers and EA among BE patients, suggesting that pathways involving inflammatory biomarkers present a potentially modifiable risk factor.

Blood-based markers of chronic systemic inflammation may also reflect systemic response to other exposures that predispose to esophageal cancer, such as obesity. Inflammatory cytokines including IL-6 and TNF-α, and acute phase proteins like CRP have been observed to be systematically elevated in obesity,(51, 52) and dietary intervention studies have shown that weight loss reduces CRP levels among obese individuals.(53) We have previously reported a modest increase in EA risk associated

with measures of central adiposity including waist-to-hip ratio and waist circumference.(23) Results from the present study indicate that CRP and IL-6 may be predictive in EA development even after adjustment for confounding effects of obesity in BE patients. Taken together, these results suggest that inflammation markers may mediate the association of obesity with EA, but they also have some independent effect on EA development beyond their effect through obesity.

This study has several strengths. Its prospective design allowed for measurement of multiple markers of inflammation and oxidative stress prior to the development of cancer, minimizing the possibility of reverse causality. For the majority of the biomarkers, we also were able to assess plasma levels at two time points during follow-up, thus reducing random measurement error and the potential for regression dilution bias.(54, 55) In addition, laboratory personnel were blinded as to the disease status of the participants. Comprehensive measurement of covariates such as WHR, pack years of smoking and use of NSAIDs enabled us to characterize these behaviors adequately and limit confounding.

Our study is limited by the relatively small number of incident EA cases, despite being one of the largest existing cohorts of BE patients. Although we controlled for potential confounding effects of smoking, obesity and NSAID use in multivariable analyses, we cannot exclude the possibility of residual confounding by measured and unmeasured risk factors. In particular, we did not collect data on *Helicobacter pylori* status, which has been shown to decrease EA risk (56, 57), while at the same time being associated with systemic inflammation(58) such that H.pylori eradication therapies reduce the blood levels of pro-inflammatory cytokines including CRP.(59) We attempted

to limit measurement error by measuring plasma biomarker levels using high sensitivity and reliable assays. However, we cannot exclude the possibility of errors in biomarker measurement resulting from degradation of biological samples during storage. Finally, as the study cohort represents a specialty clinic, results presented in the current report should be cautiously interpreted in terms of their generalizability to the general population.

In conclusion, our results indicate that CRP and to some extent IL-6 can predict progression to EA in persons with BE, especially among males. Soluble TNF receptors and isoprostanes were not found to be associated with increased EA risk. Our findings with CRP and IL6 are consistent with the literature that supports the role of chronic inflammation in the development of cancer, and EA in particular. Additional analyses involving further follow-up of this and other cohorts are needed to confirm these findings, as well as to evaluate the utility of biomarker assessment in clinical prediction and risk stratification of EA.

Table 1: Baseline characteristics of all participants, males and females in the Seattle Barrett's Esophagus Study (SBES) cohort (n = 411)

Variable	All participants (n = 411)		Males (n = 334)		Females (n = 77)	
	N	%	N	%	N	%
Age (yrs)						
30-44.9	30	7.3	25	7.5	5	6.5
45-54.9	97	23.6	83	24.9	14	18.2
55-64.9	110	26.8	84	25.2	26	33.8
65-74.9	114	27.7	93	27.8	21	27.3
≥ 75	60	14.6	49	14.7	11	14.3
Race						
White	397	96.6	324	97.0	73	94.8
Non-white	14	3.4	10	3.0	4	5.2
BMI (kg/m^2)						
≤ 25	56	13.6	41	12.3	15	19.5
25.1-≤ 30	194	47.2	167	50.0	27	35.1
30.1 - ≤ 35	125	30.4	102	30.5	23	29.9
>35	36	8.8	24	7.2	12	15.6
Cigarette smoking€						
Current	40	9.7	28	8.4	12	15.6
Former	223	54.3	192	57.5	31	40.3
Never	148	36.0	114	34.1	34	44.1
NSAID use†						
Current	169	41.1	145	43.4	24	31.2
Former	79	19.2	58	17.4	21	27.3
Never	162	39.4	130	38.9	32	41.6
Alcohol intake (drinks/day)						
0	76	18.5	52	15.6	24	31.2
≤ 1	142	34.6	108	32.3	34	44.2
2	108	26.3	93	27.8	15	19.5
≥ 3	85	20.7	81	24.3	4	5.2

N = Number/frequency, BMI = Body mass index, NSAID = Non-steroidal anti-inflammatory drugs

† NSAID use was defined as having taken NSAID at least once a week for six months or longer, NSAID use history for one male participant was missing.

€ Cigarette smoking was based on whether participants smoked 1 cigarette/day for 6 months or longer currently or in the past or never.

Table 2: Distribution of mean biomarker levels and waist-hip ratio overall and by gender in the SBES cohort. (n= 411)

	All participants (n = 411)			Males (n = 334)			Females (n = 77)		
	N	**Median**	**IQR**	**N**	**Median**	**IQR**	**N**	**Median**	**IQR**
CRP[¶] (mg/L)	398	1.90	0.95-3.60	329	1.80	0.90-3.35	69	2.95	1.7-4.35
IL-6[‡] (pg/ml)	407	1.88	1.34-3.06	332	1.77	1.31-2.87	75	2.62	1.48-3.79
sTNF- Receptor I[‡] (ng/ml)	407	1.39	1.14-1.65	332	1.42	1.15-1.66	75	1.31	1.08-1.61
sTNF- Receptor II[‡] (ng/ml)	407	5.35	4.71-6.44	332	5.34	4.68-6.40	75	5.49	4.74-6.62
Isoprostanes[£] (pg/ml)	390	53	41-74	320	51	40-66	70	74	47-107
Waist-Hip ratio[†]	409	0.95	0.91-0.99	332	0.96	0.93-1.00	77	0.87	0.81-0.91

N = Number/frequency, IQR = Inter-quartile range, CRP = C-reactive protein, IL6 = Interleukin-6, sTNF-RI & II = soluble tumor necrotic factor receptor I & II

¶ CRP was not measured in 4 people due to exhausted baseline plasma. CRP levels over 10 mg/L were excluded as per an apriori hypothesis. 4 people had plasma measured only at one time point and it was greater than 10 mg/L, 5 people had both CRP measurements greater than 10 mg/L.

‡ IL-6 and TNF receptors were not measured in 4 people due to exhausted baseline plasma samples

£ Isoprostanes were not measured in 16 individuals due to exhausted baseline plasma samples, 5 samples were not successfully measured by the laboratory.

† Two male participants had missing waist and hip circumference at baseline.

Table 3: Pearson's correlation coefficients between the biomarkers and correlates of esophageal adenocarcinoma (n = 411)

	CRP	IL6	sTNF-RI	sTNF-RII	F2-isoprostanes
Age	0.08	0.18*	0.37*	0.43*	-0.03
Waist Hip Ratio	0.10*	0.08	0.13*	0.08	-0.12*
Cigarette pack years	0.23*	0.19*	0.22*	0.09	0.10
NSAID use	0.04	0.04	0.05	0.08	0.04
CRP	-	0.44*	0.18*	0.20*	0.22*
IL-6	-	-	0.21*	0.26*	0.08
sTNF-RI	-	-	-	0.63*	0.12*
sTNF-RII	-	-	-	-	0.14*
F2-isoprostanes	-	-	-	-	-

* p-value < 0.05.
CRP = C-reactive protein, IL6 = Interleukin 6, sTNF-RI & II = soluble tumor necrosis factor receptor I & I,
NSAID = Non-steroidal anti-inflammatory drugs, NSAIDs coded Non-current vs. current
Non-smokers coded as have smoked zero pack-years

Table 4: Hazard ratios (HRs) and 95% confidence intervals (CIs) for esophageal adenocarcinoma associated with markers of systemic inflammation (CRP, IL-6, sTNF-RI & sTNF –RII) and markers of oxidative stress (F2-isoprostanes) in the SBES cohort (n = 397)

Biomarker	**EA cases/ total**	**Unadjusted HR(95% CI)**	**Adjusted for age† and gender HR(95% CI)**	**Adjusted for age†, gender, WHR‡, smoking¶ and NSAID use£ HR(95% CI)**
CRP (mg/L)€				
All participants				
Median				
Below	15/197	REF	REF	REF
Above	28/194	1.83 (0.98-3.43)	**1.98 (1.05-3.73)**	1.77 (0.93-3.37)
Quartiles				
Q1 (0.1-)	6/95	REF	REF	REF
Q2 (0.9-)	9/97	1.29 (0.46-3.63)	1.18 (0.42-3.32)	1.05 (0.37-2.99)
Q3 (1.9-)	15/95	2.35 (0.91-6.06)	2.29 (0.89-5.92)	2.12 (0.81-5.56)
Q4 (3.6-)	13/99	1.90 (0.72-5.01)	2.06 (0.78-5.44)	1.55 (0.56-4.24)
*p-value trend**		***0.03***	***0.01***	***0.04***
Males				
Median				
Below	12/155	REF	REF	REF
Above	28/165	**2.31 (1.18-4.55)**	**2.21 (1.12-4.36)**	1.93 (0.96-3.89)
Quartiles				
Q1 (0.1-)	5/79	REF	REF	REF
Q2 (0.9-)	7/76	1.20 (0.38-3.77)	1.20 (0.38-3.79)	1.09 (0.34-3.49)
Q3 (1.8-)	15/84	2.74 (0.99-7.55)	2.55 (0.92-7.02)	2.33 (0.83-6.51)
Q4 (3.4-)	13/81	2.37 (0.85-6.66)	2.36 (0.84-6.62)	1.76 (0.60-5.15)
*p-value trend**		***0.01***	***0.01***	***0.05***
IL-6 (pg/ml)				
All participants				
Median				
Below	15/197	REF	REF	REF
Above	30/197	**2.06 (1.11-3.82)**	**1.95 (1.03-3.72)**	1.79 (0.93-3.43)

Quartiles								
Q1 (0.4-)		7/98	REF		REF		REF	
Q2 (1.3-)		8/99	1.14 (0.41-3.15)		0.95 (0.34-2.66)		0.82 (0.29-2.34)	
Q3 (1.9-)		19/99	**2.74 (1.15-6.52)**		2.35 (0.96-5.77)		1.93 (0.78-4.79)	
Q4 (3.1-)		11/98	1.65 (0.64-4.24)		1.40 (0.52-3.78)		1.17 (0.42-3.26)	
	*p-value trend**			*0.64*		*0.94*		*0.87*
Males								
Median								
Below		10/161	REF		REF		REF	
Above		31/161	**3.19 (1.57-6.52)**		**2.85 (1.38-5.92)**		**2.52 (1.19-5.33)**	
Quartiles								
Q1 (0.4-)		6/81	REF		REF		REF	
Q2 (1.3-)		4/80	0.67 (0.19-2.36)		0.56 (0.16-2.01)		0.53 (0.15-1.89)	
Q3 (1.8-)		18/80	**3.06 (1.21-7.71)**		2.51 (0.96-6.51)		2.04 (0.77-5.41)	
Q4 (2.9-)		13/81	2.25 (0.86-5.93)		1.77 (0.64-4.86)		1.56 (0.55-4.43)	
	*p-value trend**			*0.66*		*0.99*		*0.81*
sTNF-I (ng/ml)								
All participants								
Median								
Below		18/197	REF		REF		REF	
Above		27/197	1.62 (0.89-2.93)		1.29 (0.69-2.42)		0.99 (0.51-1.92)	
Quartiles								
Q1 (0.3-)		11/98	REF		REF		REF	
Q2 (1.1-)		7/99	0.64 (0.25-1.64)		0.56 (0.22-1.45)		0.60 (0.23-1.56)	
Q3 (1.4-)		13/98	1.24 (0.55-2.76)		0.94 (0.41-2.15)		0.87 (0.38-1.98)	
Q4 (1.7-)		14/99	1.41 (0.64-3.10)		1.02 (0.44-2.37)		0.68 (0.27-1.68)	
	*p-value trend**			*0.18*		*0.68*		*0.69*
Males								
Median								
Below		17/161	REF		REF		REF	
Above		24/161	1.56 (0.84-2.90)		1.23 (0.63-2.41)		0.95 (0.47-1.94)	
Quartiles								
Q1 (0.3-)		11/80	REF		REF		REF	
Q2 (1.1-)		6/81	0.51 (0.19-1.37)		0.47 (0.17-1.26)		0.51 (0.19-1.38)	

Q3 (1.4-)	12/80	1.10 (0.48-2.49)	0.85 (0.36-2.00)	0.73 (0.31-1.74)
Q4 (1.7-)	12/81	1.23 (0.54-2.80)	0.88 (0.36-2.14)	0.66 (0.26-1.71)
*p-value trend**		*0.30*	*0.83*	*0.57*
sTNF-II (ng/ml)				
All participants				
Median				
Below	15/197	REF	REF	REF
Above	30/197	**2.23 (1.20-4.15)**	1.90 (0.98-3.67)	1.78 (0.90-3.52)
Quartiles				
Q1 (1.6-)	8/98	REF	REF	REF
Q2 (4.7-)	7/99	0.83 (0.30-2.29)	0.78 (0.28-2.17)	0.81 (0.29-2.25)
Q3 (5.4-)	13/98	1.68 (0.70-4.05)	1.46 (0.59-3.61)	1.47 (0.59-3.70)
Q4 (6.4-)	17/99	2.44 (1.05-5.66)	1.95 (0.76-4.95)	1.75 (0.68-4.49)
*p-value trend**		*0.22*	*0.75*	*0.86*
Males				
Median				
Below	14/161	REF	REF	REF
Above	27/161	**2.18 (1.14-4.16)**	1.81 (0.91-3.62)	1.63 (0.80-3.34)
Quartiles				
Q1 (1.6-)	8/80	REF	REF	REF
Q2 (4.7-)	6/81	0.69 (0.24-1.98)	0.62 (0.21-1.80)	0.69 (0.24-2.00)
Q3 (5.3-)	12/80	1.52 (0.62-3.72)	1.27 (0.50-3.23)	1.28 (0.50-3.30)
Q4 (6.4-)	15/81	2.17 (0.92-5.14)	1.59 (0.60-4.19)	1.44 (0.54-3.83)
*p-value trend**		*0.20*	*0.78*	*0.78*
Isoprostanes (pg/ml)				
All participants				
Median				
Below	27/186	REF	REF	REF
Above	18/191	0.60 (0.33-1.09)	0.69 (0.38-1.26)	0.56 (0.30-1.03)
Quartiles				
Q1 (14-)	13/90	REF	REF	REF
Q2 (41-)	14/96	0.98 (0.46-2.09)	1.18 (0.55-2.53)	0.93 (0.43-2.02)
Q3 (53-)	10/98	0.68 (0.30-1.54)	0.76 (0.33-1.74)	0.61 (0.26-1.41)
Q4 (74-)	8/93	0.52 (0.22-1.26)	0.74 (0.30-1.85)	0.46 (0.18-1.20)

*p-value trend**		*0.34*	*0.92*	*0.41*
Males				
Median				
Below	23/143	REF	REF	REF
Above	18/157	0.74 (0.40-1.36)	0.79 (0.42-1.46)	0.64 (0.34-1.20)
Quartiles				
Q1 (14-)	11/74	REF	REF	REF
Q2 (40-)	12/79	1.03 (0.45-2.33)	1.18 (0.52-2.69)	0.98 (0.43-2.27)
Q3 (51-)	11/80	0.96 (0.42-2.21)	1.04 (0.45-2.41)	0.80 (0.34-1.88)
Q4 (66-)	7/77	0.55 (0.21-1.43)	0.66 (0.25-1.72)	0.47 (0.18-1.25)
*p-value trend**		*0.88*	*0.79*	*0.54*

† Age was modeled as a continuous variable.
‡ Waist to Hip Ratio was modeled as a continuous variable.
¶ Cigarette smoking was modeled as pack-years smoked, never smokers were assigned a pack-year value of 0.
£ NSAID use was modeled as current vs. non-current users of NSAIDs at the baseline visit.
€ CRP measurements over 10 mg/L were excluded from the analysis.
* Test for trend was based on the likelihood-ratio test associated with addition of the variable under consideration in its continuous form.

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CHAPTER 2: Intraindividual variability over time in plasma biomarkers of inflammation and effects of long-term storage

ABSTRACT

Background: Systemic measures of chronic inflammation, often based on a single blood draw, are frequently used to study the associations between inflammation and chronic diseases such as cancer. However, more information is needed on the measurement error in these markers due to laboratory error, within-person variation over time, and long-term storage.

Methods: We investigated the intraindividual variability of inflammation markers C-reactive protein (CRP), Interleukin-6 (IL-6), and soluble tumor necrosis factor receptors I & II (sTNFRI & II) in a subsample of the Seattle Barrett's Esophagus Study cohort. Two fasting blood samples were collected between 1995 and 2009 from 360 participants on average 1.8 years apart. CRP, IL-6 & sTNF receptor levels were measured by immunonephelometry, ELISA and multiplex assays, respectively. Intra- & inter-batch coefficients of variation (CVs) were estimated using blinded pooled samples within each batch. Intraclass correlations (ICCs) were computed using random effects ANOVA.

Results: Intra- & inter-batch CVs for the pooled plasma aliquots were low (2.4-8.9%) suggesting little laboratory variability. Reliability over time was excellent for sTNF receptors ($ICC_{sTNF-RI}$=0.89, $ICC_{sTNF-RII}$=0.85) and fair to good for CRP and IL-6 (ICC_{CRP}=0.55, ICC_{IL-6}=0.57). For samples stored for over 13 years, the ICCs for CRP & IL-6 were lower than for samples stored for shorter periods, but those for sTNF receptors were unaffected by storage time.

Conclusion: sTNF receptor levels are more stable within-person over time than CRP or IL-6. Long-term storage of samples appears to increase the variability of CRP and IL6 measures, while the reliability of soluble TNF receptor measures was not affected by storage time.

INTRODUCTION

C-reactive protein (CRP), an acute-phase reactant, and pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have been implicated in the development as well as progression of a variety of human diseases, such as cancer(1-3), cardiovascular disease(4, 5), and diabetes(6). Typically, studies investigating the association of inflammatory biomarkers on subsequent risk of chronic diseases utilize established cohorts with repositories of biological specimens collected prospectively, since biomarker levels will often change after disease diagnosis. Therefore specimens will often have been stored for 10 years or more before laboratory analysis. Additionally, in most studies, biological samples collected for analyses are limited in quantities and only a single biospecimen per participant may be available for analysis. Although numerous epidemiological studies have used cytokine measurements from a single blood draw as their primary exposure, it is uncertain whether a single measure of an inflammatory biomarker is reflective of an individual's average level over time. The current evidence surrounding the reliability and intraindividual variability of inflammatory biomarkers is primarily restricted to studies involving CRP and IL-6.(4, 7-21) The existing studies are limited by small sample sizes(7, 9, 10, 12-15, 17, 19, 21), and most evaluate the within-person variability over a relatively short period of a few weeks to a year.(8, 9, 12, 14, 17-19, 21) Moreover, they typically involve healthy participants with considerably lower (and sometimes undetectable) blood levels of cytokines, thus having to deal with missing data. None of these existing studies have evaluated the effect of long-term storage of blood samples on their reliability.

In this report, we analyzed the intraindividual variability over time of biomarkers of inflammation including CRP, IL-6, soluble tumor necrosis factor receptor I (sTNF-RI) and soluble tumor necrosis factor receptor II (sTNF-RII) in a subset of the Seattle Barrett's

Esophagus Study (SBES). The SBES is a large prospective cohort of Barrett's esophagus patients being followed for development of esophageal adenocarcinoma.(22) In addition, we assessed the laboratory error and the effects of long-term storage of blood samples of these biomarkers. The biomarkers studied in this report are those that were simultaneously being evaluated for their association with esophageal adenocarcinoma, as part of separate analyses.

METHODS

Study population: The parent study, the SBES, is an ongoing prospective cohort study designed to identify predictors of neoplastic progression in Barrett's esophagus that may assist in risk stratification, screening and surveillance. It comprises persons with an established histological diagnosis of Barrett's esophagus with no history of esophageal cancer. The details involving cohort recruitment, baseline protocol and follow-up procedures have been described previously.(22, 23) Briefly, at the baseline visit, participants underwent an extensive personal interview, dietary and anthropometric assessments and provided blood samples in addition to an endoscopy with biopsy as per a standard protocol.(24, 25) At subsequent follow-up visits, shorter personal interviews were completed by the participants and blood and biopsy samples were collected. Biomarkers of interest were measured at the first two time points with available blood samples (baseline and first follow-up visits for most). The mean duration between the two visits was 1.8 years and the median 1.6 years (IQR 1.3-2.0 years). The Institutional Review Boards at the University of Washington and Fred Hutchinson Cancer Research Center approved the study.

A total of 427 participants were enrolled into the SBES cohort between February 1, 1995 and September 30, 2009, 411 of whom had at least one follow-up visit completed at the time of present data analysis. Of these, 51 participants were omitted because either their stored blood had been exhausted or blood specimens were available only at a single time-point. Hence, for the purposes of the current report, we used plasma measurements of inflammation markers from the remaining 360 participants. In addition, 31 individuals had one or both of their plasma CRP levels greater than 10 mg/L; these were omitted from analysis due to an apriori hypothesis that levels over 10 mg/L are indicative of acute inflammation and should be excluded from the reliability study, as they would be from the parent study. Thus, we used plasma CRP measures

from 329 participants while all 360 participants' biomarker measurements for IL-6, sTNF-RI and sTNF-RII were utilized for data analysis.

Laboratory methods for biomarker assessment: Fasting venous blood samples were obtained from each participant, processed within 2 hours after collection and stored at -80°C until analysis. Plasma levels of CRP were measured at the Research Testing Services laboratories at the University of Washington Medical Center, Seattle, WA; while all other cytokines were measured at the PHS Biomarker lab at the Fred Hutchinson Cancer Research Center, Seattle, WA. CRP and IL-6 assays were conducted on never-thawed plasma samples while once-thawed samples were used for the sTNF receptors measurement. Plasma CRP concentrations were determined with a high-sensitivity assay using immunonephelometry (Dade Behring Inc, Deerfield, IL, USA) as per the manufacturer's instructions. The minimum detectable limit for this assay was 0.2 mg/L for CRP. All samples below the detection limit (<1%) were assigned a value of 0.1 mg/L. Plasma levels of IL-6 were assayed using the Quantikine HS human IL-6 Elisa kit (R&D Systems Inc, HS600B) according to the manufacturer's instructions. The limit of quantification for this assay was 0.156 pg/ml and none of the samples were below this level. For every participant, IL-6 samples were run in duplicate and averaged, with a median duplicate coefficient of variation (CV) of 2.7%; samples with CVs greater than 12% were re-run in duplicate and the two new measurement values were used for analysis after averaging the duplicate values. sTNF-RI and sTNF-RII were measured using the MILLIPLEX MAP Human Soluble Cytokine Receptor Panel (Millipore, Billerica, MA, HSCR-32K) according to the manufacturer's instructions. The limit of quantification for the Millipore assay was 12.2 pg/ml and all the samples were over this limit. Similar to IL-6, samples were run in duplicate with a

median duplicate CV of 4.11% for sTNF-RI and 3.37% for sTNF-RII; samples with CVs greater than 12% were re-run and the re-run values were used for analyses. For every individual, the two samples from different time points were run in the same batch for laboratory analysis, thus limiting our capability to estimate between-batch error in the ICCs.

In addition to the quality control procedures conducted by the laboratories, we included aliquots of a blinded pooled plasma sample in every batch of study samples analyzed (two aliquots per IL-6 and sTNF receptor batches, one aliquot with every CRP batch). Intra- and inter-batch CVs were calculated based on the measurements from these blinded aliquots.

Statistical analyses: Median levels of the various biomarkers studied were computed at each of the two time points along with their interquartile ranges. To assess the laboratory component of error, we estimated the intra- and inter-batch CVs for IL-6 and sTNF receptors and inter-batch CVs for CRP using the blinded pooled plasma specimens included with each batch of study samples. CVs were calculated as 100 times the ratio of the SD (σ) to the mean (μ) and expressed as a percentage; $CV = 100 \times \sigma/\mu$. ICC's and their 95% confidence intervals (95% CIs) were calculated to evaluate the total measurement error including variation within individuals over time, using random effects analysis of variance (ANOVA) models. The within ($\sigma 2W$) and between ($\sigma 2B$) subject variance from the ANOVA models was used to compute the ICCs using the formula $ICC = \sigma 2B / (\sigma 2B + \sigma 2w)$.(26) Following Rosner's definitions of reliability, ICCs $\geq$ 0.75 were considered to indicate excellent correlation, $0.4 \leq$ ICCs < 0.75 were considered to be indicative of fair to good correlation and ICCs < 0.4 were indicative of poor reproducibility.(27) Additionally, the ICC analyses were repeated after stratifying by duration between the two samples ($\leq$ 2 years, >2years) as well as by the storage time of the earlier of the two samples (i.e.

longest stored sample) used for ICC calculation (≤13 years, >13 years) to evaluate if long-term storage affected the repeatability of these inflammation markers. The cut-points for the stratified analyses were based on the median values for the duration between the two samples and the median storage time of the earlier of the two samples, respectively. Finally, we evaluated whether the actual median biomarker values of the longest stored of the two samples varied with storage time by conducting regression analyses and likelihood-ratio tests for each biomarker, and estimating the p-value associated with storage time treated as a grouped linear variable. Analyses were completed using the STATA version 12.0 statistical software (StataCorp LP, College Station, Texas, USA).

RESULTS

The baseline descriptive characteristics of our study sample are summarized in Table 1. The median age of the study participants was 61 years (IQR 53-69 years) and the majority were males (82.2%). A large proportion (40%) of the study participants were obese i.e. body mass index (BMI) greater than 30 kg/m^2, with 10% having a BMI over 35 kg/m^2. The median waist-hip ratio for the study participants was 0.95 (IQR 0.91-0.99).

The intra- and inter-batch CVs estimating assay precisions based on the blinded pooled plasma samples are reported in Table 2. As we included only one blinded pooled sample with every CRP batch, we were unable to report the intra-batch variability in CRP measurements. CVs for intra-batch variation for the different assays ranged from 2.4-5.9% while those for inter-batch variation were between 2.9-8.9%. Low CVs are indicative of less dispersion from the mean, and therefore better reproducibility.

The medians and the interquartile ranges of the various cytokines at the two time points are reported in Table 3 along with the ICCs and their 95% CI. The overall ICCs for sTNF-RI & sTNF-RII were excellent [$ICC_{sTNF\text{-}RI}$ = 0.89, 95% CI (0.87,0.91); $ICC_{sTNF\text{-}RII}$ = 0.85, 95% CI (0.82,0.88)] while those for CRP and IL-6 were fair to good [ICC_{CRP} = 0.55, 95% CI (0.47,0.62); $ICC_{IL\text{-}6}$ = 0.57, 95% CI (0.50,0.64)]. The higher the ICC for an inflammation marker, the greater the proportion of total variation in that marker can be attributed to actual differences between individuals, rather than intraindividual variability or laboratory error. We further evaluated the reliability of these cytokines after stratification by the duration between two time points at which blood samples were collected as well as by the duration the first (earliest) of the two samples was stored (Table 3). The mean duration between the two blood samples used for biomarker measurements was 1.8 years (range 0.3-8.8 years, SD 0.97). Hence, we used a cut-off of two years to stratify the duration between two samples (i.e. $\leq$ 2 years and > 2 years). The ICC values

for the two subsets stratified by duration between the two samples were similar for most of the biomarkers (The ICCs for sTNF-RII appeared to differ by duration between samples, but it is unlikely that reliability can be better over longer duration). The mean storage time for the earlier of the two samples analyzed was 12.3 years (range, 2.3-15.4 years) for CRP and 12.6 years (range, 2.5-15.5 years) for IL-6 and sTNF receptors. We used a cut-off of 13 years to stratify storage time for the longest stored samples used for analyses. The ICCs for CRP and IL-6 appeared to differ with storage time such that ICCs were lower when at least one of the two samples was stored for more than 13 years [$ICC_{CRP,\ storage > 13\ years}$ = 0.50 (95% CI 0.40-0.60) & $ICC_{IL\text{-}6,\ storage > 13\ years}$ = 0.51 (95% CI 0.42-0.61)] than for samples stored for less than 13 years [$ICC_{CRP,\ storage \leq 13\ years}$ = 0.64 (95% CI 0.54-0.75) & $ICC_{IL\text{-}6,\ storage \leq 13\ years}$ = 0.67 (95% CI 0.57-0.76)]. In contrast, the ICCs for sTNF receptors did not appear to vary by the storage time for the longest stored sample.

We also evaluated whether the median biomarker levels for the longest sample stored varied by storage duration. The median values of all the biomarkers at four separate storage time categories (≤10 years, >10-≤13 years, >13-≤14 years and >14 years) are displayed in Table 4. There was no apparent trend in biomarker levels with increasing storage time.

DISCUSSION

In this methodologic study, we evaluated the intraindividual variability of inflammation markers CRP, IL-6, sTNF-RI and sTNF-RII over 1.8 years on average, in a subsample of the SBES cohort. Comparing the biomarker concentrations at the first two available plasma samples during follow-up (usually baseline and first follow-up), we observed that the ICCs for CRP and IL-6 were fair to good while those for sTNF-RI & II were excellent, as per the criteria described by Rosner.(27) We also observed that the ICCs for sTNF-RI and sTNF-RII did not appear to differ by storage time of up to 16 years at -80°C, while those for CRP and IL-6 appeared to be lower among those participants that had at least one of the two samples stored for over 13 years, as compared to study participants with both samples stored for less than 13 years. To our knowledge, this is the first study to examine the effects of long-term storage of blood samples on the intra-individual variability and reliability of inflammation markers. Overall, these data suggest that long term storage of blood samples may increase the variability of CRP and IL6 measures with storage over 13 years, while TNF receptors seem to be largely unaffected by prolonged storage. Hence, in epidemiological studies of longer duration, it is essential to understand the variability of the biomarkers under consideration before drawing conclusions.

The overall ICCs reported in our study are fairly consistent with those reported by previous studies. Numerous studies have examined the intra-individual variability of CRP measurements, with reported ICCs between 0.58-0.76 over a wide range of time between blood draws (2 weeks-12 years).(4, 7, 8, 10, 11, 13, 17-20) The ICC for CRP in our study (0.55), although slightly lower than those reported in the past, is still indicative of good reproducibility. The ICC values for IL-6 reported in the literature are highly variable with studies reporting values as low as 0.37(9) to as high as 0.92(13) , but most values in the range of 0.5-0.8.(10, 14, 15, 17, 19, 21) We reported an ICC value of 0.57 for IL-6 which is suggestive of good

reproducibility. Only three studies have reported on the reliability of sTNF receptors(10, 13, 19) and the sample sizes of all the three studies were much smaller (n ≤65) as compared to our study. Our findings regarding ICCs of sTNF receptors I & II (0.89 & 0.85, respectively) are similar to those by Navarro *et. al* (ICCs of 0.92 and 0.90 for sTNF-RI and sTNF-RII, respectively) and suggest excellent reproducibility of sTNF receptors. Our estimates for sTNF receptors are higher than those reported by Clendenen *et. al*. and Gu *et. al*.(10, 13) However, both these reports used an assay different from the one we used for the estimation of sTNF receptor status, which may have contributed to the difference.

In most large scale prospective epidemiological studies, enrollment into the study, data collection and follow-up may span over several years; in some studies, like ours, it may even span over decades. Such studies rely on their biological banks where samples are stored for multiple years before analysis. It is important to understand the effects of long-term storage of biological samples on their reliability, a question that is, to our knowledge, as yet unexplored with respect to markers of inflammation. We examined ICCs of the inflammation markers after stratifying on the duration of the first of the two samples analyzed for every individual and found that even after more than 13 years of storage the ICCs suggested good to excellent reproducibility. There did not appear to be any difference in the ICCs for the sTNF receptors between the two groups suggesting that these markers were largely unaffected by the duration of storage. ICCs for CRP and IL-6 appeared to decrease somewhat for the group with over 13 years of sample storage (CRP 0.50, IL-6 0.51) as compared to the group with less than 13 years of storage (CRP 0.64, IL-6 0.67). However, when we examined the median biomarker levels for the earlier of the two samples collected, we did not find any evidence of a statistical trend with increasing storage time. These results suggest that there is some concern regarding increased

variability (i.e. lower repeatability as measured by ICC's) for CRP and IL-6 over prolonged storage, however, there does not seem to be any systematic increase or decrease in the actual biomarker levels over storage time.

Overall, the ICC's computed in our study reflect several sources of measurement error: the laboratory error, the field procedures error in collection of samples (e.g. error due to variation in time between subjects before specimens reached freezer), some amount of storage error as well as individual variation over time. The only error that we were unable to capture as a part of our ICCs is the batch to batch error, as samples from the same individual were tested in the same batch. In addition to estimating the ICCs, we also evaluated the amount of laboratory measurement error or batch-to-batch variability in inflammation marker measurements by including blinded pooled plasma samples with every batch of biomarker analyzed. Little variability was found in the inflammation marker measurements in pooled aliquots within as well as between the different batches. The intra- and inter-batch CVs for the blinded pooled plasma samples were low (range, 2.4-8.9%), indicative of good reproducibility of the assays with low batch-to-batch variation.

Our study has a number of strengths, the most important one being its large sample size. To date, this is the largest study assessing the intra-individual variability of the sTNF receptors. Additionally, it is the first study to evaluate the effects of long-term storage of blood specimens on the reliability of inflammation markers. Another strength of our study is the use of never-thawed samples for most biomarker measurements and minimal processing times (<2 hours) after blood collection, both of which minimize the coefficient of variation and the laboratory error. This is particularly important for cytokines due to the concern of ex-vivo expression of cytokines from leukocytes due to prolonged blood processing times after collection.(28) Both a

strength as well as a limitation of our study was a large proportion of obese individuals (40%) in the cohort. The study participants might have had elevated levels of inflammation markers as compared to the general population due to their obesity.(29) On the one hand, this was beneficial as, unlike many of the previous studies, we did not encounter the problem of a large proportion of biomarkers being below detection limit. On the other hand, the higher ICCs observed in this report may have been the result of a higher variation in the biomarker levels of the cohort, due to a higher proportion of obese individuals.(30, 31) Another limitation of our study was that all the participants in our cohort had Barrett's esophagus, and hence our results may not be readily generalizable to other populations.

In conclusion, we observed that some inflammation biomarkers were better than others in terms of their reproducibility. The reliability of CRP and IL-6 appears to be affected with prolonged storage of blood samples, while sTNF receptors seem to be largely unaffected by long-term blood storage of up to 16 years. Hence, in epidemiological studies of prolonged duration involving assessment of biomarkers from stored samples, it is important to understand the variability and reliability of the biomarkers being studied before drawing any conclusions from hypotheses involving biomarker measurement.

Table 1: Characteristics of the study participants in the Seattle Barrett's Esophagus Study (n= 360)

Characteristic	%	Median (IQR)
Age (years)		61 (53-69)
Gender		
Male	82.2	
Female	17.8	
Body Mass Index (kg/m^2)		
≤ 25	12.5	
25.1-≤ 30	47.5	
30.1 - ≤ 35	30.6	
>35	9.4	
Waist Hip Ratio℄		
Q1 (0.72-<0.91)		0.88 (0.85-0.89)
Q2 (≥0.91-<0.95)		0.93 (0.92-0.94)
Q3 (≥0.95-<0.99)		0.97 (0.96-0.98)
Q4 (≥0.99)		1.01 (1.00-1.04)

IQR = Interquartile Range, Q1-4 = Quartiles 1-4
℄=Two participants had missing waist & hip circumferences

Table 2: Intra- & Inter-batch Coefficients of variations for blinded pooled plasma samples

Analyte	N	Intra-batch CV for pooled plasma samples (%)	Inter-batch CV for pooled plasma samples (%)
CRP	21	-	2.88
IL-6	22	4.13	4.35
sTNF-RI	22	5.87	8.93
sTNF-RII	22	2.39	6.09

CV = Coefficient of variation, CRP = C-reactive protein, IL-6 = Interleukin-6, sTNF-RI = soluble tumor necrosis factor receptor I, sTNF-RII = soluble tumor necrosis factor receptor II

Table 3: Temporal reliability of biomarkers of inflammation overall, by duration between the two samples and by storage time for the longest stored sample.

Analyte	N	Biomarker levels		ICC (95%CI)
		First sample Median(IQR)	Second sample Median(IQR)	
CRP (mg/L)[£]				
Overall	329	1.8 (0.9-3.5)	1.8 (0.9-3.6)	0.55 (0.47,0.62)
Duration between two samples (years)				
≤ 2	237	1.9 (1.0-3.5)	1.8 (0.9-3.5)	0.52 (0.43-0.62)
>2	92	1.8(0.7-3.5)	1.8 (0.7-4.25)	0.60 (0.47-0.73)
Storage time for the first sample (years)				
≤ 13	118	1.75 (0.9-3.2)	1.7 (0.8-2.9)	0.64 (0.54-0.75)
>13	211	1.9 (1.0-3.8)	1.9 (0.9-4.0)	0.50 (0.40-0.60)
IL-6 (pg/ml)				
Overall	360	1.79 (1.15-3.04)	1.87 (1.25-2.98)	0.57 (0.50,0.64)
Duration between two samples (years)				
≤ 2	256	1.80 (1.19-2.88)	1.83 (1.28-2.93)	0.54 (0.45-0.62)
>2	104	1.65 (1.12-3.16)	1.99 (1.17-3.42)	0.62 (0.50-0.74)
Storage time for the first sample (years)				
≤ 13	129	1.58 (1.12-2.74)	1.82 (1.13-2.98)	0.67 (0.57-0.76)
>13	231	1.85 (1.20-3.17)	1.88 (1.29-2.94)	0.51 (0.42-0.61)
sTNF-RI (pg/ml)				
Overall	360	1370 (1125-1650)	1366 (1116-1672)	0.89 (0.87,0.91)
Duration between two samples (years)				
≤ 2	256	1383 (1132-1660)	1382 (1132-1672)	0.90 (0.88-0.92)
>2	104	1330 (1116-1601)	1349 (1078-1688)	0.88 (0.84-0.92)
Storage time for the first sample (years)				
≤ 13 years	129	1343 (1119-1609)	1344 (1108-1608)	0.90 (0.87-0.94)
>13 years	231	1375 (1125-1682)	1375 (1120-1714)	0.89 (0.86-0.92)

sTNF-RII (pg/ml)				
Overall	360	5366 (4615-5360)	5361 (4619-6505)	0.85 (0.82,0.88)
Duration between two samples (years)				
≤ 2	256	5355 (4639-6385)	5329 (4627-6471)	0.83 (0.80-0.87)
>2	104	5429 (4549-6373)	5455 (4588-6585)	0.88 (0.84-0.93)
Storage time for the first sample (years)				
≤ 13	129	5485 (4599-6451)	5435 (4689-6410)	0.87 (0.83-0.91)
>13	231	5350 (4627-6384)	5307 (4609-6547)	0.84 (0.80-0.88)

CRP = C-reactive protein, IL-6 = Interleukin-6, sTNF-RI = soluble tumor necrosis factor receptor I, sTNF-RII = soluble tumor necrosis factor receptor II, ICC = Intraclass correlation coefficient, IQR = Interquartile range, 95% CI = 95% confidence interval, £ = 31 participants had one or both of their CRP values greater than 10 mg/L, and were omitted from analysis

Table 4: Distribution of biomarkers of inflammation by storage time of the first sample

Biomarker	Median (IQR) of biomarker levels by storage time of first sample (years)								p-trend*
	≤ 10		>10-≤13		>13-≤ 14		>14		
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
CRP (mg/L)	47	1.11 (0.7-2.9)	71	2.1 (1.1-3.4)	77	1.8 (0.9-3.6)	134	2.05 (1.0-3.8)	0.58
IL-6 (pg/ml)	54	1.21 (1.0-2.92)	75	1.94 (1.29-2.73)	76	1.88 (1.31-3.26)	155	1.85 (1.11-3.14)	0.63
sTNF-RI (pg/ml)	54	1322 (1116-1503)	75	1439 (1131-1672)	73	1467 (1196-1803)	158	1354 (1114-1565)	0.09
sTNF-RII (pg/ml)	54	5413 (4485-6603)	75	5485 (4599-6296)	73	5522 (4961-6741)	158	5289 (4532-6073)	0.20

CRP = C-reactive protein, IL-6 = Interleukin-6, sTNF-RI = soluble tumor necrosis factor receptor I, sTNF-RII = soluble tumor necrosis factor receptor II, IQR = Interquartile range
* Test for trend was based on the likelihood-ratio test associated with storage time treated as a grouped linear variable, adjusted for confounding effects of age, gender and obesity as captured by the baseline waist-hip ratio.

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CHAPTER 3: Inflammation and obesity markers in relation to telomere length in persons with Barrett's esophagus

ABSTRACT

Background: Telomeres shorten with every cell division, ultimately leading to programmed cell death. Telomere shortening has been consistently associated with older age and male gender. Inflammation has also been implicated in cell senescence and may promote telomere shortening in chronic conditions such as obesity and diabetes. However, little is known about the relationship between inflammation markers and leukocyte telomere length (LTL).

Methods: We examined the correlates of telomere length in a cross-sectional analysis of individuals with Barrett's esophagus. LTL was measured using PCR in peripheral leukocytes of 295 individuals. Data on lifestyle variables, including obesity and smoking, were collected using personal interviews. Markers of inflammation and oxidative stress, including C-reactive protein, interleukin-6, soluble tumor necrosis factor receptors I & II (sTNF-RI & II), and F2-isoprostanes were measured in addition to obesity- and diabetes-related biomarkers. Associations with LTL were tested with linear and logistic regression models after adjustment for confounders.

Results: LTL was significantly associated with age ($r = -0.3$, $p < 0.001$), gender ($r = 0.14$ for females, $p = 0.01$) and cigarette pack-years ($r = -0.11$, $p = 0.04$). Odds of having short LTL were significantly higher for participants in the highest tertile for sTNF-RI (Adjusted OR = 2.19; 95% CI 1.00-4.85, p-trend = 0.02). LTL was not significantly associated with any other lifestyle factors or biomarkers measured.

Conclusion: Increasing age, male gender, smoking history and sTNF-RI levels were associated with short LTL among persons with Barrett's esophagus but no correlations were observed between LTL and other inflammatory markers or measures of obesity. In order to further establish the potential relationships between inflammation markers and telomere length, larger longitudinal studies are necessary.

INTRODUCTION

Telomeres are the extreme ends of chromosomes, consisting of a large number of tandem DNA repeats of the sequence TTAGGG/CCCTAA bound to a variety of proteins.(1) They vary in length from two to approximately fifty kilobase pairs (kbp) and play a role in chromosomal protection, nuclear attachment and replication.(2, 3) Except immortal, neoplastic, and germ cells, the telomeres of all other cells are shortened by every cell division.(4) When telomere shortening on a particular chromosome reaches a critical threshold, a DNA damage checkpoint mechanism may be initiated and the cell may stop replicating, leading to senescence and biological ageing.(1, 2) Hence, telomere length has been considered as a biomarker for ageing.(5)

Telomere shortening has consistently been associated with older age(6, 7), male gender(6, 7) and Caucasian race.(8) Shorter telomeres have also been shown to be associated with lifestyle factors such as smoking(9, 10), obesity(9, 10), and lack of physical activity(11), but these associations have not been consistent across all the studies.(12) Telomere shortening has been established as a risk factor for numerous age-related diseases such as cardiovascular disease(7), diabetes(13), and chronic renal disease.(14) It can also be viewed as an anti-neoplastic mechanism aimed at countering the accumulation of oncogenic mutations. Hence, errors in the telomere shortening processes potentially could produce neoplastic cells.(15) Indeed, telomere length abnormalities have been observed early in precursor lesions for cancer in various tissues.(16) Additionally, epidemiological studies have established shortened telomeres as potential biomarkers of cancer incidence and mortality(17-20), evidence being strongest for cancers of the bladder(21), esophagus(22), and stomach(23).

Inflammation and oxidative stress have been associated with ageing in general(24), and shortening of telomeres in particular.(14, 25) Oxidative stress and presence of reactive oxygen species may cause single-stranded breaks in telomeric DNA either directly or indirectly through

improper DNA repair owing to their high content of guanines.(26) Chronic conditions such as obesity and diabetes may also affect telomere length by promoting inflammation. Indeed, obesity studies have implicated adipokines in the development of insulin resistance, and in turn accelerated cell senescence and ageing.(27) Although it is hypothesized that inflammation may contribute to telomere shortening by accelerating cell turnover(28), little is known about the relationship between various systemic markers of inflammation, obesity and diabetes, and leukocyte telomere length (LTL).

In the current cross-sectional analysis, we sought to investigate the correlates of telomere length among a subset of the Seattle Barrett's esophagus study (SBES) cohort. We evaluated the correlations between telomere length and demographic factors including age and gender, lifestyle factors such as smoking and obesity and a set of biomarkers, including systemic markers of inflammation and markers of obesity and diabetes. As persons with Barrett's esophagus (BE) have significant underlying systemic inflammation, this cohort provided a good opportunity to understand the relationship between inflammation and LTL.

METHODS

Study population: The SBES is a prospective cohort study aimed at identifying factors that predict neoplastic progression in persons with BE, a precursor to esophageal adenocarcinoma.(29) Participants underwent a personal interview at enrollment along with dietary and anthropometric assessments, endoscopy with biopsies and collection of blood samples, details of which have been previously described.(30, 31) Periodic follow-up visits were conducted at which participants were endoscoped, blood samples were collected and demographic and risk factor information was updated. A total of 427 participants were enrolled into the SBES between February 1, 1995 and September 30, 2009. For the purposes of this report, we used baseline data on 295 individuals that had LTL measurements available at their baseline visit to test cross-sectional associations between LTL and demographic and lifestyle factors. Of the 295 with available LTL measurements, 202 had blood biomarker measurements available at baseline while 32 had blood biomarkers measured within a year from their baseline visit. We used biomarker data from these 234 individuals to test the associations between LTL and markers of inflammation, diabetes and obesity. Participants with C-reactive protein (CRP) levels greater than 10 mg/L (n = 12) were omitted from analysis involving CRP due to an apriori hypothesis that levels over 10 mg/L are indicative of acute inflammation and are therefore not indicative of the participants' long-term inflammation level. Institutional Review Boards at the University of Washington and Fred Hutchinson Cancer Research Center approved the study.

Anthropometric measurements, including body mass index (BMI), waist circumference (WC) and waist-hip ratio (WHR) were measured as described previously.(30) Cigarette use was described as ever use (at least one cigarette/day for six months or longer) and cigarette pack-years of smoking (number of cigarette packs smoked per day times the number of years smoked). Alcoholic drinks consumption was computed after combining responses for beer, wine and

liquor consumption. A history of non-steroidal anti-inflammatory drugs (NSAID) and statin use was also collected at baseline, as reported previously.(31, 32)

Biomarker assessment: SBES participants provided fasting venous blood samples which were processed within 2 hours after collection and stored at -80°C until analysis. Intra- and inter-assay coefficients of variation (CVs) were calculated by including blind duplicate samples with each batch of biomarker measurement. Inflammation markers measured and their intra- and inter-batch CVs are respectively: high sensitivity CRP (hs-CRP; immunonephelometry; Dade Behring Inc, Deerfield, IL; inter-batch CV 2.88%), interleukin-6 (IL-6; Quantikine HS human IL-6 Elisa kit; R&D Systems Inc, Minneapolis, MN; CVs 4.13% and 4.35%), soluble tumor necrosis factor receptor- I & II (sTNFR-I & II; MILLIPLEX MAP Human Soluble Cytokine Receptor Panel; Millipore, Billerica, MA; sTNFR-I CVs 5.87% and 8.93%, sTNFR-II CVs 2.39% and 6.09%), F2-isoprostanes (gas chromatography/ negative ion chemical ionization mass spectrometry; 6890N Agilent gas chromatograph coupled to a 5973 quadruple mass spectrometer in negative ion mode; Santa Clara, CA; assay precision ±3% and accuracy 97%). We also measured several obesity- and diabetes-related markers including leptin, adiponectin, glucose, and insulin on stored blood samples, the details of which have been described previously.(33) Homeostatic model assessment (HOMA) score was computed from the insulin and glucose measurements as previously described.(33) The laboratory personnel were blinded to other exposures and outcomes.

LTL Analysis: Genomic DNA was extracted from buffy coat samples stored at -80°c using the salting out procedure.(34) Telomere length was measured using quantitative polymerase chain reaction (PCR) on an Applied Biosystems 7900HT thermocycler in a 384-well format.(35) Telomeric DNA was amplified along with a single-copy control gene (36B4) that acted as an

internal control. A relative measure of telomere length, T/S ratio, was computed by dividing the amount of telomeric DNA (T) with the amount of single-copy control gene (S). All measurements were performed in triplicate and the median of the three measures was used for all calculations. In addition, two internal control DNA samples were run within each plate to evaluate inter-plate variation. For T/S ratios, the intra- and inter-assay CVs were 6% and 7%, respectively. The mean T/S ratio of the cohort was standardized to 1.0 to enable comparisons within the cohort.

Statistical Analysis: Medians and standard deviations for continuous variables and proportions for categorical variables were computed separately by telomere tertiles for all participants. Age- and gender-adjusted correlations between various participant characteristics, including biomarker levels, and LTL were investigated with Pearson product-moment correlations as well as linear regression models. As the distributions of telomere length and the various biomarkers measured were confirmed to be normal, we used all the variables in their original form. We further assessed whether selected participant characteristics and higher levels of inflammation and obesity biomarkers were associated with increased odds for short telomere length (T/S ratio < 0.735). The biomarkers were evaluated as continuous measures as well as by grouping participants into biomarker tertiles. For every variable of interest, three different models were run: unadjusted, age- and gender-adjusted, and further adjusted for major correlates of inflammation: smoking (cigarette pack years)(36) and obesity (measured as waist circumference).(37, 38) Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using unconditional logistic regression models. Tests for trend were based on the likelihood-ratio test associated with addition of the variable under consideration in its continuous form. Sensitivity analyses were conducted after dropping those that had their biomarkers measured

within a year of their baseline visit and results are noted where different. Results with p-values less than 0.05 were considered to be statistically significant. All statistical analyses were carried out using STATA statistical software (version 12.0; Stata Corp, College Station, TX).

RESULTS

Participant characteristics within telomere tertiles are shown in Table 1 along with results of the correlation and linear regression analysis. Average age of the cohort was 60.9 years and most were overweight (average BMI = 29.2 kg/m^2, 38% with BMI >30 kg/m^2). The cohort was comprised predominantly of males (80%) and current or past smokers (66%). At baseline just over a third (36%) of the cohort were regularly using NSAIDs, and about 10% were current statin users.

Gender-adjusted LTL was inversely correlated with age ($r = -0.30$, $p < 0.0001$) with an attrition rate of 0.003 ± 0.001 per year in the T/S ratio. Statistically significant associations were found with gender ($p = 0.01$) such that age-adjusted LTL was on an average 0.04 ± 0.03 units longer in females as compared to males. There was little evidence of an association between telomere length and any of the obesity-related variables such as BMI, WHR or waist circumference in the linear regression models. Cumulative smoking exposure measured as cigarette pack-years smoked was negatively correlated with LTL ($r = -0.11$, $p = 0.04$). The regression coefficient of pack-years smoked remained statistically significant even after adjustment for age and gender. Other lifestyle variables such as alcohol and use of medications such as NSAIDs and statins were not found to be associated with telomere length. Further adjustment for confounding effects of smoking and obesity did not change the results for any of the participant characteristics evaluated in table 1 (data not shown).

Table 2 displays the correlations and linear associations between the various biomarkers and LTL. No significant association with telomere length was observed with obesity-related biomarkers such as leptin and adiponectin, or diabetes-associated biomarkers such as glucose, insulin or HOMA score. All of the inflammation biomarkers were inversely correlated with LTL, although none significantly so. Linear regression models adjusted for age and gender also did not

reveal significant associations between telomere length and any of the inflammation biomarkers measured. Further adjustment for correlates of inflammation, including smoking (cigarette pack-years) and obesity (waist circumference) did not alter any of the above results (data not shown).

To identify any hidden non-linear associations between the biomarkers and short telomeres, we examined whether higher levels of the biomarkers were associated with increased odds of short (lowest tertile) LTL in logistic regression models (Table 3). Additionally, we also evaluated the odds of short LTL for selected participant characteristics and lifestyle factors. Each one year increase in age increased the odds of having short telomeres by 4% (Adjusted OR = 1.04, 95% CI 1.02-1.07). Females were significantly less likely to have short telomeres than males (Adjusted OR = 0.37, 95% CI 0.18-0.78). None of the obesity-related variables such as BMI, WHR or waist circumference or smoking variables such as ever use and cigarette pack years were associated with short LTL in logistic regression models. We observed that the odds of having short telomeres were significantly higher for those participants having high levels of soluble TNF receptor I (Adjusted OR = 2.30, 95% CI 1.11-4.78). This OR estimate did not change much even after excluding those individuals with cytokine measurements within 1 year after the baseline visit rather than at the baseline visit itself (Adjusted OR = 2.32, 95% CI 0.98-5.46). Additionally, a significant trend was observed with increasing levels of sTNF-RI such that those in the highest tertile for the inflammation marker were at more than twice increased odds of having short telomeres as compared to those in the lowest tertile for the biomarker (Adjusted OR = 2.19, 95% CI 1.00-4.85, p-trend = 0.02). This trend remained significant even after dropping those with cytokine measurements within one year after baseline visit from the analysis (p-trend = 0.04). Although the unadjusted odds for short LTL were significant for sTNF-RII, this association disappeared after adjustment for confounders. There were no significant associations

observed between short LTL and higher levels of CRP, IL-6 or F2-isoprostanes. Similar analyses on obesity- and diabetes-related biomarkers did not show any significant results (data not shown).

DISCUSSION

In this cross-sectional study we found evidence linking a serum-based marker of inflammation, elevated soluble TNF receptor I, with shortened LTL. Individuals with sTNF-RI levels in the highest tertile were at 2.2 times increased odds of having short LTL compared to those individuals with sTNF-RI levels in the lowest tertile. We also found suggestive evidence of a trend of short LTL with increasing sTNF-RII levels. Additionally, as expected, we also observed associations between LTL and age, gender and smoking.

Our findings are in line with previous research regarding associations of LTL with individual and lifestyle characteristics. A recent review by Sanders *et. al.* summarized the utility of telomere length as a biomarker for ageing in epidemiological studies and established age and gender to be most consistently associated with LTL across studies.(12) In that review, the authors presented evidence from 13 studies looking at the association of LTL with age, all of them showed that increasing age is significantly associated with short LTL. Furthermore, five studies that have evaluated the relationship of LTL with gender have all reported statistically significant association of shorter telomeres among males.(12) Out of these studies, the only study that reported linear associations with increasing T/S ratio and gender (the Multi-Ethnic Study of Atherosclerosis cohort) had β-coefficients very similar to ours.(39) The evidence regarding smoking has been mixed.(12) Some studies have reported a positive association with LTL(40), while others have reported negative association.(6, 7, 39) There was no correlation between the epidemiological or statistical methods used in these studies or particular study populations, and the likelihood of a positive association.

To date, there are limited data on the association between inflammatory markers and LTL. Reports that have observed an association between increasing levels of inflammatory markers and short LTL are few in number.(7, 14, 25) One hypothesis that may explain such a

cross-sectional association between LTL and inflammation markers suggests that inflammation may promote and accelerate telomere attrition by enhancing leukocyte turnover and replicative senescence.(41, 42) Shorter telomeres may also cause programmed cell death and lead to accumulation of excessive senescent cells within the body which in turn may be responsible for the elevated levels of inflammation markers.(28, 43) A factor supporting this hypothesis is that the main sources for TNF-α secretion and thereby shedding of the soluble TNF receptors, fibroblasts and mononuclear cells, are both involved in clearing of the senescent cells from the body.(44) In the present analysis, we only found associations with LTL for sTNF receptors, particularly sTNF-RI, and not CRP, IL-6 or F2-isoprostanes. Moreover, this association was apparent only in logistic regression models (no association was found in linear models). We are not sure of the robustness of this finding with sTNF-RI as the cut-points for both short telomeres and the sTNF receptor tertiles were arbitrary, and this significant finding with sTNF-RI may be entirely data driven. As this was a cross-sectional analysis, directionality of the LTL-inflammation association cannot be inferred, and further evaluation in a longitudinal setting is necessary to better understand the relationship between telomere length and markers of inflammation.

Very few studies have looked at the association of telomeres with obesity-related biomarkers such as leptin and adiponectin,(45-47) or diabetes-related biomarkers insulin, glucose or HOMA.(47-49) The rationale behind these studies is similar to that behind studies of the inflammation-LTL relationship i.e. the presence of these chronic conditions may give rise to increased turnover of cells, and ultimately shorter telomeres. Results from previous studies show positive associations with some obesity and diabetes markers and negative associations with others, that do not seem to be patterned on statistical or measurement techniques, or populations

studied. Results from this report did not show a significant association of LTL with any of the obesity- or diabetes-related biomarkers.

Strengths of our study include a well characterized study sample with detailed information on important confounders, fairly reliable biomarker and LTL laboratory measurements and their blinded assessments. There are several limitations as well. The cross-sectional design of this study does not allow us to make any temporal interpretations. The sample size is also limited, thus meriting the need for longitudinal observations in larger cohorts evaluating the link between inflammation markers and LTL. There is also a possibility that the association observed with sTNF-RI might be a chance finding as we conducted multiple statistical tests in the limited sample size available. The study participants were a high-risk cohort of Barrett's esophagus patients, many being obese (38%). This might have resulted in higher levels of inflammation markers among the study participants as compared to the general population.(37) This can be viewed upon as both a strength and a limitation. On the one hand, we did not encounter the problem of a large proportion of biomarkers being below detection limit, while on the other hand, the higher inflammation levels in this cohort make the results from this study less generalizable to other populations. Although we controlled for confounding effects of age, gender, smoking and obesity in multivariable analysis, there still might be a possibility of residual confounding by other measured or unmeasured confounders. In addition, we cannot exclude the possibility of measurement errors in estimation of the various biomarkers evaluated in this study. Assuming that such measurement errors, if present, are non-differential, they could only have driven the risk estimates towards the null.

In summary, the present study shows that the inflammation marker, sTNF-RI, and possibly sTNF-RII are associated with short LTL among persons with BE. In addition, we

confirmed previous findings of shortened telomeres with increasing age, male gender and increasing pack-years of smoking. We did not find any association between short telomeres and obesity or obesity-related biomarkers. In order to further establish the potential relationship between inflammation and telomere length, larger longitudinal studies are needed.

Table 1: Seattle Barrett's Esophagus Study participant characteristics in relation to leukocyte telomere length (n=295)

Sample characteristic		**n**	**Leukocyte Telomere Length Tertile**			**Pearson's Correlation Coefficient[¶]**	**Linear Regression**	
			Short (n = 96)	**Middle (n= 99)**	**Long (n = 100)**		**Age & Gender adjusted Beta (95% CI)**	**p-value**
Leukocyte Telomere Length (T/S ratio)†		295	0.68 (0.05)	0.78 (0.03)	0.90 (0.08)	-	-	-
Age(per year) †		295	65.5 (10.9)	62.0 (12.0)	57.0 (10.8)	**-0.30[£]**	**-0.003 (-0.004,-0.002)[£]**	**< 0.001**
Gender ‡	Males	236	84 (87.5)	78 (78.8)	74 (74.0)	-	REF	-
	Females	59	12 (12.5)	21 (21.2)	26 (26.0)	-	**0.04 (0.01,0.07)[€]**	**0.01**
BMI(per kg/m^2) †		290	28.1 (3.6)	29.2 (4.5)	29.1 (4.3)	0.05	0.002 (-0.002,0.005)	0.35
Waist-Hip Ratio (per unit) †		294	0.95 (0.06)	0.96 (0.07)	0.95 (0.07)	0.05	0.10 (-0.13, 0.34)	0.38
Waist Circumference (per inch) †		294	39.2 (4.0)	39.6 (4.9)	39.6 (4.4)	0.09	0.002 (-0.001, 0.005)	0.14
Cigarette use ‡	Never	100	33 (34.4)	29 (29.3)	38 (38.0)	-	REF	-
	Ever	195	63 (65.6)	70 (70.7)	62 (62.0)	-	-0.007 (-0.03, 0.02)	0.64
Cigarette pack-years (per pack-year) †		195	34.0 (25.6)	23.6 (24.8)	18.5 (19.1)	**-0.11**	**-0.0006 (-0.0011,-0.0000)**	**0.04**
Alcoholic drinks/day (per drink) †		240	1.7 (4.9)	1.4 (7.9)	1.0 (2.5)	-0.07	-0.002 (-0.004, 0.001)	0.20
NSAID use ‡	Non-current	190	58 (60.4)	64 (64.7)	68 (68.0)	-	REF	-
	Current	105	38 (39.6)	35 (35.3)	32 (32.0)	-	-0.001 (-0.029, 0.027)	0.93
Statin use ‡	Non-current	265	83 (86.5)	92 (92.9)	90 (90.0)	-	REF	
	Current	30	13 (13.5)	7 (7.1)	10 (10.0)	-	-0.023 (-0.066, 0.020)	0.29

† = Median (Standard deviation), ‡ = Frequency (%), £ = Adjusted only for gender, ¶ = Adjusted for age & gender
BMI = Body mass index, NSAID = Non-steroidal anti-inflammatory drugs, CI = Confidence interval
Leukocyte telomere length tertile: Short < 0.735, Middle 0.735-0.846, Long ≥ 0.847

Table 2: Biomarker concentrations in relation to leukocyte telomere length (n=234)

Biomarkers		Leukocyte Telomere Length Tertile*			Pearson's Correlation Coefficient¶	Linear Regression	
	n	Short (n = 76)	Middle (n= 80)	Long (n = 78)		Age & Gender adjusted Beta (95% CI)	p-value
C-reactive protein (mg/L)	222	2.1 (2.3)	1.8 (2.5)	2.3 (2.1)	-0.04	-0.002 (-0.009, 0.005)	0.57
Interleukin-6 (pg/ml)	234	2.1 (2.7)	2.0 (2.9)	1.7 (1.6)	-0.10	-0.005 (-0.011, 0.001)	0.11
sTNF-RI (ng/ml)	234	1.5 (0.5)	1.3 (0.4)	1.3 (0.5)	-0.06	-0.016 (-0.050, 0.020)	0.36
sTNF-RII (ng/ml)	234	5.6 (1.6)	5.3 (1.5)	5.0 (1.1)	-0.003	-0.003 (-0.012, 0.011)	0.96
F2-isoprostanes (pg/ml)	224	54.0 (34.2)	57.5 (26.5)	54.0 (38.3)	-0.04	-0.0002 (-0.0006, 0.0003)	0.53
Leptin (ng/ml)	287	7.8 (10.3)	9.5 (12.3)	10.1 (10.3)	-0.01	-0.0001 (-0.002, 0.002)	0.90
Total Adiponectin (mcg/ml)	226	5.2 (2.8)	5.4 (2.4)	4.4 (2.5)	-0.01	-0.001 (-0.007, 0.006)	0.88
HMW Adiponectin (mcg/ml)	226	1.9 (1.7)	2.0 (1.6)	1.8 (1.6)	0.01	0.001 (-0.010, 0.011)	0.90
Glucose (mg/dL)	226	91.0 (14.6)	91.5 (28.4)	93.0 (30.3)	0.04	0.0002 (-0.0004, 0.0008)	0.52
Insulin (uU/ml)	226	7.2 (5.2)	6.4 (7.7)	6.9 (5.4)	-0.001	-0.0001 (-0.0024, 0.0024)	0.99
HOMA score	226	1.6 (1.3)	1.5 (3.3)	1.6 (1.7)	0.01	0.0003 (-0.006, 0.007)	0.93

¶ = Adjusted for age & gender, *Median (standard deviation)

sTNF-RI & RII = Soluble Tumor necrosis factor receptor I & II, HMW = High molecular weight, HOMA score = Homeostatic Model Assessment score

Leukocyte telomere length tertile: Short < 0.735, Middle 0.735-0.846, Long ≥ 0.847

Table 3: Odds ratios (OR) for short* leukocyte telomere length by subject characteristics and markers of inflammation

Characteristic	N	Unadjusted OR (95% CI)	Age & gender adjusted OR (95% CI)	Age, gender, smoking£ & obesity€ adjusted OR (95% CI)
Age (per year)	295	**1.05 (1.03,1.07)**	**1.05 (1.03,1.08)** £	**1.04 (1.02,1.07)** £
Gender (Females vs. males)	295	**0.46 (0.23,0.92)**	**0.43 (0.21,0.87)** €	**0.37 (0.18,0.78)** €
BMI (per kg/m^2)	290	**0.91 (0.85,0.97)**	0.93 (0.87,1.00)	0.96 (0.86,1.07)
Waist circumference (per inch)	294	0.96(0.91,1.02)	0.94(0.88,1.00)	0.94 (0.88,1.00)
Waist-hip ratio (per unit)	294	1.11(0.77,1.60)	0.72(0.45,1.16)	0.92 (0.49,1.74)
Cigarette use (Ever vs. never)	295	1.01 (1.00,1.03)	1.01 (0.99,1.03)	0.99 (0.96,1.03)
Pack-years continuous (per pack-year)	295	**1.01 (1.00,1.02)**	1.01 (1.00,1.02)	1.01 (1.00,1.02)
Pack-years Tertiles				-
Non-smokers		REF	REF	REF
T1 (0.05-)		0.58 (0.28,1.20)	0.64 (0.30,1.35)	0.64 (0.30,1.37)
T2 (14-)		0.91 (0.47,1.76)	0.79 (0.40,1.57)	0.84 (0.42,1.70)
T3 (36-)		1.58 (0.83,3.01)	1.30 (0.66,2.56)	1.40 (0.70,2.77)
p-trend†		*0.04***	*0.27*	*0.20*
CRP continuous (mg/L)	222	0.98 (0.87,1.11)	0.95 (0.82,1.09)	0.98 (0.84,1.13)
CRP Tertiles				
T1 (0.1-)		REF	REF	REF
T2 (1.1-)		1.36 (0.67,2.74)	1.40 (0.66,2.96)	1.69 (0.78,3.68)
T3 (2.9-)		0.91 (0.45,1.85)	0.81 (0.37,1.78)	1.04 (0.45,2.40)
p-trend†		*0.74*	*0.44*	*0.74*
IL-6 continuous (pg/ml)	234	1.06 (0.95,1.17)	1.02 (0.90,1.15)	1.04 (0.92,1.18)
IL-6 Tertiles				
T1 (0.4-)		REF	REF	REF
T2 (1.5-)		1.45 (0.74,2.86)	1.21 (0.58,2.50)	1.25 (0.59,2.62)
T3 (2.6-)		1.25 (0.63-2.47)	1.07 (0.50,2.31)	1.22 (0.55,2.71)
p-trend†		*0.32*	*0.77*	*0.51*
sTNF-RI continuous (ng/ml)	234	**3.30 (1.75,6.23)**	**2.02 (1.02,3.99)**	**2.30 (1.11,4.78)**
sTNF-RI Tertiles				
T1 (0.3-)		REF	REF	REF
T2 (1.2-)		1.14 (0.56,2.33)	0.90 (0.42,1.91)	1.00 (0.46,2.15)
T3 (1.5-)		**3.25 (1.64,6.43)**	1.81 (0.85,3.84)	**2.19 (1.00,4.85)**
p-trend†		*<0.001***	*0.04***	*0.02***
sTNF-RII continuous (ng/ml)	234	**1.30 (1.07,1.58)**	1.08 (0.86,1.35)	1.06 (0.84,1.33)
sTNF-RII Tertiles				
T1 (2.1-)		REF	REF	REF
T2 (4.9-)		0.90 (0.44,1.82)	0.64 (0.30,1.37)	0.68 (0.32,1.47)
T3 (6.0-)		**2.52 (1.29,4.90)**	1.42 (0.66,3.04)	1.45 (0.67,3.15)
p-trend†		*0.01***	*0.50*	*0.62*
F2-isoprostanes continuous (pg/ml)	224	1.00 (0.99,1.01)	1.00 (1.00,1.01)	1.01 (1.00,1.02)
F2-isoprostane Tertiles				
T1 (14-)		REF	REF	REF
T2 (48-)		0.62 (0.31,1.25)	0.67 (0.32,1.41)	0.72 (0.34,1.52)
T3 (67-)		0.93 (0.48,1.83)	1.28 (0.60,2.71)	1.55 (0.71,3.39)
p-trend†		*0.88*	*0.31*	*0.18*

BMI = Body mass index, CRP = C-reactive protein, IL-6 = Interleukin-6, sTNF-RI & RII = Soluble Tumor necrosis factor receptor I & II, CI = Confidence interval

£ - Adjusted for cigarette pack-years smoked at baseline, € - Adjusted for waist circumference at baseline, £ = Adjusted only for gender, € = Adjusted only for age

*Short Leukocyte telomere length defined as lowest tertile (T/S ratio < 0.735), ** p-value significant at 0.05 level

† Test for trend based on the likelihood-ratio test associated with addition of the variable under consideration in its continuous form.

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CONCLUSION

In this dissertation project, we primarily sought to understand the relationship between chronic inflammation, oxidative stress and the development of esophageal adenocarcinoma (EA) in a prospective cohort of persons with Barrett's esophagus (BE). We utilized data from the Seattle Barrett's Esophagus Study (SBES) cohort to investigate the associations between systemic markers of chronic inflammation and oxidative stress and EA through detailed statistical analysis. As a part of this dissertation, we also assessed the intra-individual variability of the inflammation markers that we had measured. Additionally, we also evaluated the correlates of telomere length, another marker hypothesized to capture a person's long-term level of inflammation and oxidative stress(1), among persons with BE.

We were able to add to the extensive body of research implicating inflammation in the development of cancers. Our results indicated that systemic markers of chronic inflammation, particularly C-reactive protein (CRP) and to some extent interleukin-6 (IL-6) can predict progression to EA in persons with BE. Specifically, we observed a two-fold increased risk for EA development among those with mean CRP levels above the median value of 1.9 mg/L compared to those with values below the median (p-trend for continuous CRP = 0.01). We also observed a two-fold increased risk among persons with IL-6 values above the median. We did not find any association between EA and soluble tumor necrosis factor receptors I & II (sTNF receptors I & II) or F2-isoprostanes, a marker of oxidative stress. The findings with CRP and IL-6 are consistent with existing literature supporting a role of inflammation in cancers at other sites, including lung, colon and breast.(2-4)

A known risk factor for progression to EA among BE patients is obesity, particularly central adiposity.(5) Obesity is also considered to be a state of low-grade systemic inflammation

with elevated systemic levels of markers such as CRP and IL-6.(6, 7) In a previous analysis involving the SBES cohort, we have shown modest associations between measures of central adiposity such as waist-hip ratio and waist circumference and development of EA.(8) In this dissertation project, we show that CRP and IL-6 can predict the increased risk of EA among BE patients even after adjustment for the confounding effects of obesity. These observations taken together suggest that inflammation may mediate the association between obesity and EA, but it also has some independent effect on progression to EA beyond its effect through obesity. Further analysis, specifically a mediation analysis involving inflammation and obesity, may have to be undertaken to understand the exact contribution of inflammation in the obesity-EA pathway.

As part of this dissertation project, we also evaluated the intra-individual variability and reliability of markers of inflammation, including CRP, IL-6 and sTNF receptors. We observed that sTNF receptor levels are more stable within-person over time than CRP or IL-6. Prolonged storage of samples prior to laboratory analysis affected the reliability of CRP and IL-6 while sTNF receptors were unaffected by such a long-term storage. These observations underline the importance of taking into account the long-term within-person variations in biomarker levels, especially in the interpretation of results from epidemiological studies of prolonged duration involving the use of inflammation markers.

For the final project in this dissertation, we investigated the correlates of telomere length among BE patients in a cross-sectional analysis. Increasing age, male gender, smoking history and sTNF-RI levels were associated with short telomeres among persons with BE but no correlations were observed with other inflammatory markers or obesity-related biomarkers. In a previous analysis in the SBES cohort, we have reported an association between shorter leukocyte telomere length and increased risk of progression to EA.(9) Telomere length is also hypothesized

to be a measure of a person's underlying level of inflammation and oxidative stress.(1) Factors such as insulin resistance, obesity and smoking, that may cause low-grade inflammation and oxidative stress, have been shown to reduce leukocyte telomere length.(10, 11) Taken together, these observations suggest that short telomere length may be a correlate of the underlying level of inflammation in persons with BE. In order to further establish the potential relationships between inflammation markers and telomere length, larger longitudinal studies will have to be undertaken.

In summary, ours is one of the first studies in the expanding literature linking inflammation to esophageal cancer, where we have demonstrated an association between pre-diagnostic elevated levels of inflammation markers and EA development. Additional analyses involving further follow-up of the SBES and other cohorts are needed to confirm and replicate these findings. It is crucial to understand the complex biological mechanisms involving inflammation and telomeres among persons with BE, so as to be able to evaluate the utility of biomarker assessment in clinical prediction and risk stratification of EA. If less invasive techniques could assess risk of EA onset among BE patients, prevention efforts could then be targeted to the subset of patients with the highest risk; substantially reducing cost, patient anxiety, and potential morbidity.

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