

## Reliability of Serum Biomarkers of Inflammation from Repeated Measures in Healthy Individuals

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### Abstract

**Background:** Biomarkers of low-grade systemic inflammation are used to study the associations of inflammation with chronic diseases, including cancer. However, relatively little is known about the intraindividual variability of most of these measures.

**Methods:** Fasting serum samples, collected at baseline and the end of  $\geq 3$ -week washout periods in a four-diet crossover feeding trial, were used to measure the inflammatory markers high sensitivity C-reactive protein (hsCRP), interleukin (IL)-6, tumor necrosis factor (TNF)- $\alpha$ , IL-8, and soluble TNF receptor (sTNFR) I and II. Participants included 62 men and women for analyses of IL-6 and CRP and 56 for analyses of IL-8, TNF- $\alpha$ , and sTNFRs, aged 20 to 40, who were free of factors known to influence inflammation, for example, chronic disease, medication use, heavy alcohol use, smoking, and obesity (body mass index  $>30$  kg/m<sup>2</sup>). Intraclass correlations (ICC) were estimated using random effects ANOVA, across all four time points ( $\sim 6$  weeks apart).

**Results:** ICCs for TNF- $\alpha$  and sTNFR I and II were very high: ICC = 0.92 [95% confidence interval (CI), 0.89–0.96], 0.92 (95% CI, 0.88–0.95), and 0.90 (95% CI, 0.85–0.94), respectively. ICCs for IL-8 and hsCRP were 0.73 (95% CI, 0.63–0.83) and 0.62 (95% CI, 0.49–0.75), respectively. The ICC for IL-6 was considerably lower, ICC = 0.48 (95% CI, 0.36–0.62). Three measures of IL-6 would be needed to achieve a reliability coefficient (Cronbach  $\alpha$ ) of 0.75.

**Conclusions:** With the exception of IL-6, reliability of all inflammatory markers in our panel was high.

**Impact:** This suggests that a single measure accurately captures the short-term (e.g., 4–6 months) variability within an individual. *Cancer Epidemiol Biomarkers Prev*; 21(7): 1167–70. ©2012 AACR.

### Introduction

Cytokines, chemokines, acute-phase proteins, and other soluble factors are involved in the inflammatory process and cell survival, growth, and proliferation (1). Circulating serum markers of inflammation are increasingly being used as biomarkers of low-grade systemic inflammation associated with risk of chronic diseases, such as rheumatoid arthritis (2), cardiovascular disease (3), and cancer (4). However, a relatively small number of studies have been published on the intraindividual variability (i.e., test-retest reliability) of most of these measures, with the exception of C-reactive protein (CRP) and interleukin (IL)-6, for which a large literature exists (5–17). The majority of existing reliability studies have examined samples at only 2 different time points, typically years apart. In addition, many of these studies are limited by small sample sizes or inclusion of participants with chronic

conditions that may affect the inflammatory status of an individual. The primary aim of this investigation was to determine the intraindividual variability of 6 biomarkers of inflammation [i.e., high sensitivity (hs)CRP, IL-6, tumor necrosis factor (TNF)- $\alpha$ , IL-8, soluble TNF receptor (sTNFR) I and II] in serum across 4 time points within a 6-month time span, in a well-characterized population of healthy individuals. Results from this study will aid in selection of inflammatory biomarkers in intervention and population studies.

### Materials and Methods

#### Research design and study participants

The study activities were conducted using archived samples from a completed study, "Enzyme Activation Trial 2" (2EAT), and carried out at Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, as described previously (18). The 2EAT study was a randomized, controlled, crossover feeding trial of *Brassica* and *Apiaceous* vegetables. Recruitment, enrollment, feeding, and sample collection took place between March 2003 and July 2007; laboratory analysis for this project took place in 2010. Each diet was consumed for 14 days with a minimum of a 3-week washout period between the diets, for a total of 5 to 6 months participation in the study. Day 0

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serum from each diet period was used for the present study. Eligibility criteria included men and women from the greater Seattle area who were healthy and between the ages of 20 and 40 years. Exclusions were made for respondents with health conditions known to influence biotransformation enzyme activity and inflammation, such as chronic disease, medication use, heavy alcohol consumption, smoking, and obesity (body mass index (BMI) > 30 kg/m<sup>2</sup>).

Analyses for 2 panels of inflammatory biomarkers, (i) hsCRP and IL-6; and (ii) TNF- $\alpha$ , IL-8, and sTNFR I and II, were carried out separately. A total of 62 participants were included in the analyses for hsCRP and IL-6. Not all participants completed all time points. The total number of participants per time point (T) was:  $n = 62$  for T1 and T2;  $n = 59$  for T3; and  $n = 48$  for T4. Samples were available for 56 individuals (27 men and 29 women) for analyses of IL-8, TNF- $\alpha$ , and sTNFR I and II. The total number of participants at each time point was:  $n = 56$  for T1;  $n = 52$  for T2;  $n = 47$  for T3; and  $n = 25$  for T4. The study was approved by the Institutional Review Board at the FHCRC, and all participants gave informed consent.

### Specimen collection

Biologic samples were collected during each 2-week feeding period at days 0 and 14 in the morning after a 12-hour overnight fast (19). Tubes without additive were allowed to clot at room temperature for 30 minutes before they were centrifuged to separate the serum. Serum was aliquoted and stored at  $-80^{\circ}\text{C}$ . Day 0 serum collections for each diet period were used for the present study. The average length of time between sampling time points was 6 weeks.

Serum IL-6 concentrations were assayed using Quantikine high-sensitivity human IL-6 ELISA kit (R&D Systems, Inc.) according to manufacturer's instructions. The limit of quantification (LOQ) was 0.156 pg/mL. Serum hsCRP was measured using CRP Ultra Wide Range reagent (Genzyme Diagnostics) on a Roche Cobas Mira chemistry analyzer and read at 570 nm. The LOQ for this assay in our laboratory was 0.2 mg/L. IL-8 and TNF- $\alpha$  were multiplexed and assayed using the high-sensitivity human cytokine panel, and the sTNFRs using the human soluble cytokine receptor panel (Millipore). LOQs were 0.548 and 12.2 pg/mL, respectively. Samples were run in duplicate, and the median duplicate intraassay coefficients of variation (CV) were: 5.1% for IL-6, 5.9% for hsCRP, 6.3% for IL-8, 9.1% for TNF- $\alpha$ , 2.3% for sTNFR I, and 2.1% for sTNFR II. A blinded pooled serum sample was included in each batch to track plate-to-plate variation. The interplate CVs were 2.9% for IL-6, 3.1% for hsCRP, 15.3% for IL-8, 16.9% for TNF- $\alpha$ , 11% for sTNFR I, and 6% for sTNFR II. The assays were conducted on never-thawed samples with the exception of hsCRP and the sTNFRs, for which once-thawed samples were used. All samples from the same individual for all diet periods were run on the same plate in duplicate. Other than 49

observations for hsCRP (of 248 total observations), none of the samples were below the limit of detection.

Samples were stored between 3 and 7 years before analyses. There was no indication that storage time was associated with degradation of any samples in adjusted regression models (partial  $R^2$  ranged from 0.009–0.02 for all markers, data not shown). Health status was monitored using daily records which tracked, among other things, participant illness. If a participant reported illness before the start of a study period, they did not begin until the illness resolved; if the participant became ill during a study period, it was noted in the participant's chart.

### Statistics

All inflammatory biomarker data were log-transformed before analysis to normalize distributions. Biomarker data are presented as geometric means and 95% confidence intervals (95% CI). A random effects analysis of variance model was used to estimate the intraclass correlations (ICC) and 95% CI across all 4 time points. We use the interpretation by Rosner (20), where ICC values between 0.4 and 0.75 indicate fair to good correlation, and values of 0.75 or greater indicate excellent correlation. To reduce the potential for inclusion of samples with unreported illness, outliers that fell 5 SDs above the median, and greater than a distance of 2 SDs from any other data point, were entered as missing. For hsCRP values below 0.2 mg/L (20% of the observations, the majority from 11 individuals), multiple imputation using 10 imputed data sets with values between 0.01 and 0.19, with a mean and median of 0.1 was applied to estimate the ICC (21), and an adjusted standard error was used to calculate the 95% CI. For measures with an ICC below 0.5, we calculated the number of repeated measures that would be needed to yield a reliability (Cronbach  $\alpha$ ) of  $\geq 0.75$ . All analyses were conducted using the StataSE, v12.0 (StataCorp) statistical platform. All statistical tests are 2-sided and a  $P$  value < 0.05 was considered statistically significant.

### Results

Table 1 gives the demographic characteristics and first blood draw (time 1) serum concentrations of the inflammatory biomarkers for the study participants. ICC coefficients using all 4 time periods are given in Table 2. Because we used diet periods for participants who did not complete all 4 diets, there are fewer observations at the third and fourth time points, respectively. ICC coefficients for TNF- $\alpha$  and the soluble TNF receptors I and II were excellent (ICC  $\geq 0.90$ ). The ICCs for IL-8 (ICC = 0.73) and hsCRP (ICC = 0.62) were good, whereas the reliability for IL-6 was fair (ICC = 0.48). Three measures of IL-6 would need to be averaged to achieve a reliability coefficient (Cronbach  $\alpha$ ) of 0.75.

### Discussion

In the present study, reliability of all inflammatory markers in the panel over 4 time points was good to

**Table 1.** Characteristics of study participants

<b>Demographics</b>	<b>n</b>	<b>Mean (SD)</b>
Age, y	62	30.4 (6.1)
BMI, kg/m <sup>2</sup>	62	24.2 (3.3)
Female (%)	31 (50)	
Race (%)		
Caucasian	37 (60)	
Asian	20 (31)	
Other	5 (8)	
<i>Inflammatory biomarker</i>		Geometric mean <sup>a</sup> (95% CI)
TNF- $\alpha$	56	3.0 (2.2–4.0)
sTNFR I	56	953 (859–1,058)
sTNFR II	56	4,153 (3,903–4,419)
hsCRP	62	0.5 (0.3–0.6)
IL-8	56	3.3 (2.8–3.9)
IL-6	62	1.0 (0.8–1.2)

<sup>a</sup>Geometric mean T1 serum concentrations; all concentrations are reported in units of pg/mL except for hsCRP which is reported as mg/L.

excellent with the exception of IL-6, suggesting that, for these markers, a single measure accurately reflects an individual's inflammatory status over a 4- to 6-month period.

Results for TNF- $\alpha$  (ICC = 0.92) were higher than other studies reporting ICCs ranging from 0.48 to 0.69 in healthy individuals (7, 12, 15), although the interval in the present study was shorter and time points more frequent. Very few studies have evaluated the reliability of the soluble TNF receptors. Recently, Clendenen and colleagues (12) reported ICCs of 0.31 and 0.68 for sTNFR I and II, respectively, from 2 different time points over a 2-year period in healthy women. However, the sample size was small ( $n = 18$ ) and the confidence intervals wide. Gu and colleagues (16) reported higher ICCs, 0.68 and 0.80 among 65 women, yet these values are still lower than the ICCs found in this study (>0.90). Reliability for hsCRP and IL-8 was good.

**Table 2.** ICC coefficients (95% CI) by serum inflammatory biomarker

<b>Biomarker</b>	<b>n<sup>a</sup></b>	<b>ICC</b>
TNF- $\alpha$	52	0.92 (0.89–0.96)
sTNFR I	52	0.92 (0.88–0.95)
sTNFR II	52	0.90 (0.85–0.94)
hsCRP	62	0.62 (0.49–0.75)
IL-8	50	0.73 (0.63–0.83)
IL-6	62	0.48 (0.36–0.62)

NOTE: Time points corresponding with illness were excluded.

<sup>a</sup>Number of participants that completed  $\geq 2$  time points.

Results for hsCRP are comparable with the reported ICCs in the range of 0.6 to 0.8 in various populations (5–11, 16). Investigations of the reliability of IL-8 have varied from fair to good (7, 12, 16, 17).

The reliability for IL-6 in this study was fair. This is similar to that reported by Ho and colleagues (15), but lower than other studies (7, 14, 16, 17). It is not clear why this marker performed poorly relative to the other markers in this study, in terms of intraindividual reliability. All samples for the same individual were run in the same batch, on the same plate in duplicate, therefore assay variability was likely not a considerable factor. Because this was a healthy population with very low baseline inflammatory concentrations, we hypothesize that the large number of values near the bottom of the standard curve might be contributing to the lower reliability, as a smaller variance of exposure leads to lower reliability (22). It may also be that this marker is more easily perturbed by environmental exposures than other markers of inflammation. For example, physical activity acutely raises serum IL-6 more consistently than other inflammatory biomarkers and may be one source of variability (23, 24). IL-6 has been purported to play a crucial role in the pathogenesis of many chronic inflammatory diseases, and is frequently used as a measure of inflammation in many research settings, including those of autoimmune diseases, multiple sclerosis, inflammatory bowel diseases, and many cancers (25). Therefore, further study of the reliability of this marker is warranted.

Advantages of this study include comparison of inflammatory biomarkers at 4 different time points within a 6-month period, within the context of a randomized crossover design. Multiple fasting samples were obtained for the same individual, at the same time of day, with the same length of time between sample collections. The higher reliability of most of the markers in this study than in other studies may reflect improved platform performance and sensitivity of the assays since previous investigations or more frequent measures over a shorter duration than in previous studies.

Several limitations of the present analysis should be considered. First, although all observations in the present analysis were detectable, 21% of the samples for hsCRP were below an accurate LOQ (0.2 mg/L). These lower values of serum hsCRP are not unexpected given our healthy population. While this may have had a minor effect on the ICC, it has little clinical relevance as any value below 0.2 mg/L is extremely low and would be interpreted similarly. Of interest, other than low values for hsCRP, there were not any observations that were below the limit of quantitation for any of the other markers. Another limitation is the unbalanced distribution of samples by time point. Not all participants completed all 4 diet periods resulting in fewer participants at the third and fourth diet periods. Therefore, the power to detect reliability measures was reduced. Finally, although the stringent inclusion and exclusion criteria in the parent study provided for a homogenous population and reduced

potential confounding factors, this may have also limited the generalizability of the results. Further studies of longer duration, and in other populations, are needed.

Although long-term (e.g., year-to-year) reliability was not assessed, with the exception of IL-6, reliability of all inflammatory markers in this panel was high. For most markers, evidence is provided that a single measure accurately captures the month-to-month variability within an individual. These markers may be considered reliable for short-term population and intervention studies.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** S.L. Navarro, C.Y. Wang, A.R. Kristal, M.K. Kratz, J.W. Lampe

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