# Discrimination Ratio Analysis of Inflammatory Markers: Implications for the Study of Inflammation in Chronic Disease

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**To understand the role of inflammation in chronic disease it is important to have a reliable measure of habitual inflammatory status. A number of acute-phase response markers have been used as measures of inflammatory status, but the ability of a single measure to appropriately reflect habitual inflammatory status has not been assessed. This study compares the ability of different inflammatory markers to characterize habitual inflammatory status in overweight women. A single fasting blood sample was taken from 86 overweight women (mean body mass index [BMI], 35.2 kg/m$^2$; range, 26.2 to 47.6 kg/m$^2$) and a number of inflammatory markers (both acute-phase response markers and cytokines) were measured. A randomly selected subpopulation of 15 women attended on 2 further occasions for further blood samples. Using the subpopulation, discrimination ratios (DRs) were calculated for each inflammatory marker to assess the within-subject variability. The DRs were then used to determine the relationship between these markers, adjusted for within-subject variability, in the whole population. In this highly controlled experimental environment, interleukin-6 (IL-6), with a DR of 3.71, was the cytokine with the greatest ability to discriminate between subjects, suggesting that it is best able to characterize habitual inflammatory status. Sialic acid was the acute-phase response marker with the highest DR (3.16), and showed stronger correlations with other inflammatory markers, including C-reactive protein (CRP), than IL-6. This study suggests that use of some inflammatory markers, such as CRP, with large within-individual variability, will underestimate the relationship between inflammation and disease, and thus relationships between inflammation and chronic disease may be stronger than previously appreciated. Future studies should consider IL-6 or sialic acid to provide a more robust measure of inflammatory status.**


OVERWEIGHT AND OBESITY are associated with a number of health problems, including type 2 diabetes, dyslipidemia, hypertension, stroke, and cardiovascular disease in addition to a range of other conditions.[1] There are a number of mechanisms to explain this association, but recent attention has focused on the role of adipokines, such as tumor necrosis factor-$\alpha$ (TNF$\alpha$), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1).[2] Several studies have shown associations between adiposity and a sustained, low-grade elevation in both cytokines[3] and acute-phase markers in the circulation.[4,5] The distribution of body fat is also important. Intra-abdominal or visceral fat is more metabolically active and secretes more adipokines than fat from subcutaneous adipose beds.[6,7] Recent evidence suggests higher levels of cytokines and acute-phase proteins may be associated with an increased risk of some metabolic diseases such as type 2 diabetes and cardiovascular disease, and that inflammation may be part of the causal pathway for obesity-related disease.

In cross-sectional studies, cytokines and acute-phase proteins (as markers of inflammation) have been positively associated with type 2 diabetes[8,9] and risk factors for cardiovascular disease.[10-12] More importantly, a number of prospective studies have shown that markers of inflammation predict disease in later life. Those with low-grade elevations in acute-phase markers and cytokines including IL-6, sialic acid, and C-reactive protein (CRP) have been shown to be at higher risk of subsequently developing diabetes[13-16] and cardiovascular disease.[11,17]

To examine the relationship between inflammatory status and diseases such as type 2 diabetes and cardiovascular disease, it is important to be able to measure an individuals' habitual inflammatory status as accurately as possible. The inflammatory response is a complex process involving a coordinated cascade of metabolic responses. The concentration of any one protein may not give an appropriate representation of an individual's overall inflammatory status. IL-6 is the primary stimulus of the acute-phase response, which is characterized by the production of a range of proteins from the liver. The acute-phase proteins are a large group of proteins, including CRP, $\alpha_1$-acid glycoprotein (AGP), $\alpha_1$-antichymotrypsin (ACT), fibrinogen, and PAI-1, that are involved in diverse aspects of host defense, tissue repair, and clearance of cellular debris. Different acute-phase proteins follow very different profiles in the circulation in terms of both their change from baseline and their time course, reflecting individual regulation and clearance.[18] Sialic acid (or *N*-acetylneuraminic acid) has also been used as a marker of inflammation.[13,19,20] It is not itself an acute-phase protein but rather forms the terminal component of many acute-phase proteins, including AGP and ACT. Measurements of inflammatory markers are further confounded by the biological nature of the inflammatory system with sometimes dramatic rapid fluctuations.[18] This biological variability, together with measurement errors relating to sample collection, processing, and the limits of assay and instrument sensitivity, contributes to measurement imprecision. This imprecision will diminish the relationship observed between inflammatory status and disease, suggesting that observations to date may underestimate the importance of this relationship.

This study examines the ability of a single measure of circulating inflammatory markers to characterize an individual's habitual status and, therefore, discriminate between individuals. The relationships between the markers are also exam-

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ined to determine how well each marker represents the overall inflammatory environment.

## MATERIALS AND METHODS

### *Population*

Eighty-six overweight female subjects were recruited by advertisement from the community to take part in the study at the Medical Research Council (MRC) Human Nutrition Research. The study was approved by the local research ethics committee and all subjects gave informed written consent. Female subjects, with a body mass index (BMI) greater than 25 kg/m$^2$, with no known diabetes, chronic inflammatory condition, liver disease, or malignancy were included in the study. Any subjects who had a chronic inflammatory condition or a clinically apparent acute event, defined as 3 or more of the inflammatory measures having concentrations outside 2 SD for this population, were also excluded from the analysis. All subjects attended the volunteer suite at MRC Human Nutrition Research for a single blood test and anthropometric measurements. A randomly selected subpopulation of 15 subjects was studied on 2 further occasions equally spaced over a 6-month period. All subjects were weight-stable (±5% body weight) over the 6-month period.

### *Data Collection*

Blood samples were collected between 8 and 10 AM, after a 12-hour overnight fast. Samples were iced and centrifuged at 3,000 rpm for 20 minutes within 1 hour of collection and frozen at −80°C until analyzed. Samples collected for fibrinogen analysis were kept at room temperature throughout the processing procedure to prevent cryoprecipitation of the fibrinogen before freezing the plasma sample at −80°C.

Weight was measured to the nearest 10 g using a digital scale and height to the nearest 0.5 cm using a wall-mounted stadiometer. BMI was calculated as weight (kilograms) divided by height (meters) squared. Serum sialic acid was measured by a colorimetric enzyme assay (Roche, Welwyn Garden City, UK) as previously described[21] and adapted for use on the Hitachi 912 Clinical Analyser (Roche). The interassay coefficient of variation (CV) was 0.83% at 3.09 mmol/L and 1.23% at 1.55 mmol/L. Serum AGP was measured using an immunological agglutination assay (Roche). The interassay CV was 2.3% at 80 mg/dL and 1.9% at 140 mg/dL. Plasma ACT was measured using an immunochemical assay (Dako, Denmark). The interassay CV was 5.2% at the low control and 1.8% at the high control. Serum CRP was measured using a high-sensitivity particle-enhanced turbidimetric assay (Dade-Behring, Walton, UK). The interassay CV was 1.7% at 12 mg/L. Plasma fibrinogen was measured using a thrombin-clotting method.[22] The interassay CV was 4.4%. PAI-1 was measured in iced citrated plasma using the Coatest PAI method (Chromogenix Instrumentation Laboratory, Milano, Italy). The interassay CV was 7.6% at 12 AU/mL and 1.6% at 23 AU/mL. Serum IL-6 and TNF$\alpha$ were measured using a high-sensitivity enzyme-linked immunosorbent assay (ELISA) principle (R&D Systems, Abingdon, UK). The interassay CV for TNF$\alpha$ was 5.1% at the high control, 4.6% at the medium control, and 10.8% at the low control. The interassay CV for IL-6 was 7.7% at the high control, 9.7% at the medium control, and 23.2% at the low control.

### *Statistical Analysis*

All data on inflammatory markers was $\log_e$-transformed before analysis to normalize distributions and to remove heteroscedasticity. The discrimination ratio (DR) was used to calculate an index of the ability to distinguish between individuals to compare the different measurements[23] in a subpopulation (n = 15) of the main study population (n = 86), where 3 repeat samples were taken per subject. The DR is related to the reliability coefficient, although it has the advantage of a statistical test to compare DRs from different measurements. The DR is a ratio of the between-subject SD ($SD_B$) to the within-subject SD ($SD_W$), and is calculated using the equation:

$$\mathrm{DR} = \sqrt{(MS_B - MS_W)/(k \times MS_W)}$$

where $MS_B$ = between-subject mean squares, $MS_W$ = within-subject mean squares, and $k$ = number of samples.

Ninety-five percent confidence intervals for the DR were calculated using a noncentral F-distribution, with degrees of freedom n-1 and n($k$ − 1) and noncentrality parameter (n − 1) × $k$ × DR$^2$, where n is the number of subjects. The DRs for different inflammatory markers were compared using Cochran's theorem with chi-square distribution to show that if the statistic Q exceeded 95% for r-1 degrees of freedom (where r is the number of DRs being compared) the DRs are unequal, at $P < .05$.

Pearson's correlation coefficients were calculated between the inflammatory markers for the whole study population (n = 86). Ninety-five percent confidence intervals for the correlation coefficients were calculated using Fisher's method.[24] Since a subpopulation was used to calculate the DR for each measurement, it is appropriate to use the DR to adjust the Pearson's correlation coefficients for attenuation by within-subject variation[23] using:

$$\eta = \sqrt{(\kappa_1 \times \kappa_2)}$$

so that

$$r_{adj} = \frac{r}{\eta}$$

where $\eta$ = attenuation factor, $\kappa$ = reliability coefficient for the measurements being compared, $r$ = Pearson correlation coefficient, and $r_{adj}$ = adjusted Pearson correlation coefficient.

## RESULTS

All women were overweight, with a mean BMI of 35.0 kg/m$^2$ (range, 26.2 to 47.6 kg/m$^2$) and a mean age of 44 years (range, 21 to 69 years). Baseline values for each of the inflammatory markers are shown in Table 1. There were no significant differences between the characteristics of the whole study population (n = 86) and the subpopulation (n = 15) at baseline. BMI was significantly correlated with each of the inflammatory markers ($P < .05$) except TNF$\alpha$ ($P = .59$). Age was not significantly correlated with any of the inflammatory markers.

The DR was calculated for the acute-phase markers ACT, AGP, CRP, fibrinogen, PAI-1, and sialic acid, and the cytokines IL-6 and TNF$\alpha$ (Fig 1). Of the acute-phase markers, sialic acid, with a DR of 3.16, has the highest DR, showing that it is best able to discriminate between individuals ($\chi^2$, $P < .05$). The DR for CRP was only 2.23. Of the cytokines, IL-6, with a DR of 3.71, has a higher DR than TNF$\alpha$ ($\chi^2$, $P < .05$).

Since there were no significant differences in any of the inflammatory markers between the subpopulation and the whole population, the DR can be applied to the whole study population. To assess the relationship between inflammatory markers, Pearson's correlation coefficients and 95% confidence intervals were calculated between each of the inflammatory markers and the markers with the highest DRs, IL-6, sialic acid, and the commonly used acute-phase protein, CRP (Table 2). Using the DR calculated in the subpopulation, correlation coefficients were then adjusted for the attenuation attributable to measurement imprecision, to show the true underlying relation-

**Table 1. Baseline Inflammatory Characteristics of the Whole Study Population (N = 86) and Subpopulation (n = 15)**

| | Whole Population (N = 86) | | | Subpopulation (n = 15) | | |
|---|---|---|---|---|---|---|
| | Mean | SD | Range | Mean | SD | Range |
| ACT* (g/L) | 0.3 | 0.07 | 0.1-0.5 | 0.3 | 0.05 | 0.2-0.4 |
| TNFα* (pg/mL) | 1.7 | 2.7 | 0.0-18.9 | 1.8 | 1.6 | 0.4-7.0 |
| Fibrinogen* (mg/dL) | 347.4 | 70.4 | 217.0-562.0 | 334.4 | 44.8 | 259.0-412.0 |
| PAI-I* (AU/mL) | 18.7 | 10.9 | 1.0-38.7 | 19.3 | 9.6 | 3.82-32.7 |
| CRP* (mg/dL) | 3.8 | 6.3 | 0.6-23.4 | 2.8 | 2.7 | 0.7-8.9 |
| AGP* (mg/dL) | 93.8 | 21.7 | 62.8-152.7 | 89.9 | 14.8 | 65.7-114.5 |
| Sialic acid* (mmol/L) | 2.3 | 0.3 | 1.7-3.1 | 2.2 | 0.3 | 1.9-2.7 |
| IL-6* (pg/mL) | 2.9 | 3.6 | 0.3-21.7 | 2.5 | 1.5 | 0.6-5.4 |

*Log-transformed for analysis. Geometric mean presented for transformed data. No significant differences between whole study population and subpopulation.

ship between the inflammatory markers (Table 2). Sialic acid showed stronger correlations with other inflammatory markers than IL-6.

## DISCUSSION

To assess the relationship between habitual inflammatory status and disease in epidemiological studies, it is necessary to have a simple, reproducible and representative index of inflammatory status. In this analysis, sialic acid is the most stable marker of the acute-phase response and is best able to discriminate between individuals. Sialic acid also correlates well with other components of the acute-phase response. This is perhaps not surprising since sialic acid is not itself an acute-phase protein, but the terminal glycoprotein found as part of many acute-phase proteins. Together, these glycoproteins explain approximately 70% of the total sialic acid concentration.[25] Thus, measuring serum sialic acid provides an integrated measure of the inflammatory response, which is less prone to day-to-day variability of individual markers, and may be useful when defining habitual status. While there have been previous studies of inflammatory marker variability,[26-28] no study has examined the variability of sialic acid, compared such a wide range of inflammatory markers or used the DR analysis.

In this study the inflammatory markers are significantly correlated with each other though the correlation coefficients are not particularly high. The reasons for the weakness of these relationships are likely to be multiple. During the acute-phase response, the concentrations of many proteins increase several-fold above their basal levels. However, this increase is not uniform in either magnitude or duration, indicating that different acute phase proteins may be individually regulated with regard to expression, metabolism, and clearance. Furthermore, as demonstrated by this analysis, intra-individual variability is high for some markers, introducing additional imprecision into the estimates of the association.

In this analysis, IL-6 was best able to discriminate between individuals (DR = 3.71), but it did not correlate as well with other inflammatory markers as sialic acid (Table 2). Sialic acid correlated significantly better with CRP, fibrinogen, and AGP, than IL-6. CRP has been widely adopted for cross-sectional and

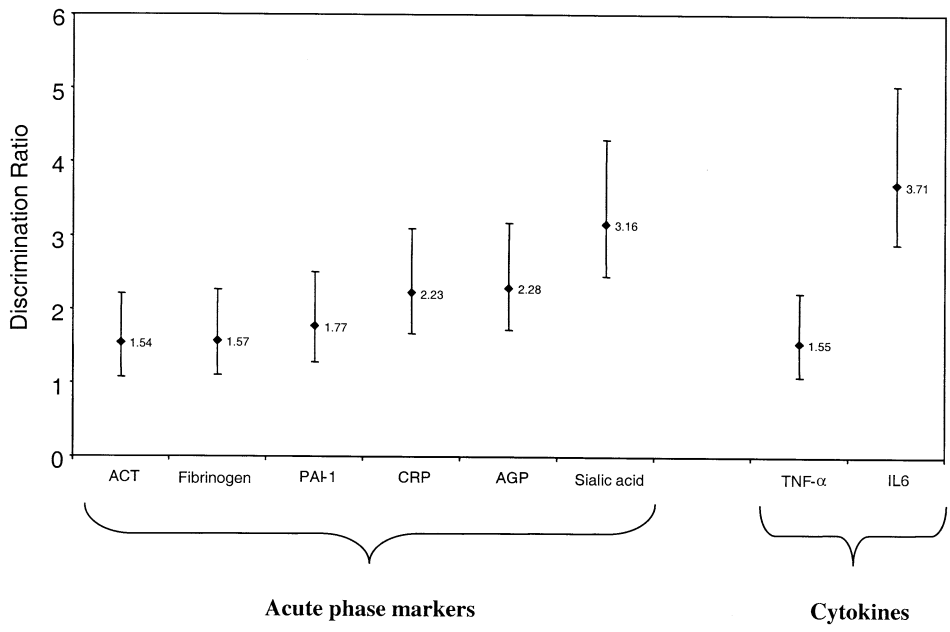


**Fig 1. DR for each inflammatory marker (error bars represent 95% confidence interval for DR).**

**Table 2. Pearson's Correlation Coefficients and Confidence Intervals Between Different Inflammatory Markers (N = 86)**

| | IL-6 | Adjusted IL-6 | Sialic Acid | Adjusted Sialic Acid | CRP | Adjusted CRP |
|---|---|---|---|---|---|---|
| BMI | 0.454 (0.28, 0.61) | | 0.345 (0.14, 0.52) | | 0.554 (0.39, 0.69) | |
| IL-6 | | | 0.302 (0.01, 0.48) | 0.311 (0.11, 0.49) | 0.382 (0.19, 0.55) | 0.400 (0.21, 0.56) |
| Sialic acid | 0.302 (0.10, 0.48) | 0.311 (0.11, 0.49) | | | 0.662 (0.52, 0.77)§ | 0.695 (0.57, 0.79)§ |
| CRP | 0.382 (0.19, 0.55) | 0.400 (0.21, 0.56) | 0.662 (0.52, 0.77)† | 0.695 (0.57, 0.79)† | | |
| Fibrinogen | 0.302 (0.10, 0.48) | 0.325 (0.12, 0.50) | 0.550 (0.38, 0.68)† | 0.596 (0.44, 0.72)† | 0.519 (0.35, 0.66) | 0.571 (0.41, 0.70)‡ |
| AGP | 0.279 (0.07, 0.46) | 0.291 (0.08, 0.47) | 0.533 (0.36, 0.67)† | 0.559 (0.39, 0.69)† | 0.355 (0.15, 0.53) | 0.378 (0.18, 0.55) |
| TNF$\alpha$ | 0.210 (0.00, 0.40) | 0.227 (0.02, 0.42) | 0.176 (−0.04, 0.37) | 0.191 (−0.02, 0.39) | 0.105 (−0.11, 0.31) | 0.116 (−0.10, 0.32) |
| PAI-1* | 0.255 (0.04, 0.45) | 0.272 (0.06, 0.46) | 0.037 (−0.18, 0.25) | 0.040 (−0.17, 0.25) | 0.209 (−0.01, 0.41) | 0.227 (0.01, 0.42) |
| ACT* | 0.289 (0.08, 0.47) | 0.312 (0.11, 0.49) | 0.343 (0.14, 0.52) | 0.372 (0.17, 0.54) | 0.346 (0.14, 0.52) | 0.382 (0.18, 0.55) |

NOTE. Pearson's correlation coefficients adjusted for attenuation by within-subject variation, calculated using the DR. Data displayed as correlation coefficient (95% confidence interval).

*Data skewed by 2 subjects, excluded from the calculation of the correlation coefficients so that Pearson's correlation coefficients could be calculated and adjusted by the DR

†Correlation with inflammatory variable significantly different between IL-6 and sialic acid, $P < .05$.

‡Correlation with inflammatory variable significantly different between IL-6 and CRP, $P < .05$.

§Correlation with inflammatory variable significantly different between IL-6 and CRP, $P < .001$.

prospective studies assessing the relationship between habitual inflammatory status and disease,[15,17,29,30] but this study suggests that sialic acid is an appropriate and potentially superior tool for this purpose.

The applicability of this study is limited by the selection of a specific population in which the DR was calculated. Although this is important in order to apply the DR to attain accurate adjusted correlation coefficients, the DR for different inflammatory markers may be different for different populations. The small sample size selected for intra-individual variability study also limits the study. The nature of the inflammatory response, with the potential for acute events, makes it difficult to determine habitual levels. Individuals with known chronic inflammatory conditions or evidence of an acute event were excluded. This tends to improve the DR and the correlation coefficients between inflammatory markers but is important in order to assess the true association between raised habitual inflammatory status and the risk of metabolic disease as opposed to acute fluctuations caused by intercurrent infection.

The choice of marker must reflect the experimental hypotheses, but there are also practical considerations. Cytokines are more difficult to measure than many acute phase markers, even in studies with highly controlled conditions and sample collection procedures and in this analysis the CVs for IL-6 were considerably higher than those for sialic acid and CRP. These differences may be amplified as part of a large-scale epidemiological study, where highly controlled experimental conditions are harder to achieve, with the effect of introducing greater measurement error.

At present it is unclear whether acute-phase proteins represent a causal element in the pathogenesis of disease or simply act as a marker of cytokine activity. Certainly both cytokines and acute-phase proteins are useful markers of chronic low-grade inflammation. In some studies (although not all) acute-phase proteins show better correlations with, or are better predictors of, disease than cytokines. Ridker et al used inflammatory markers to predict cardiovascular events at 3-year follow up.[11] They showed that for the highest versus lowest quartile of IL-6 the risk of cardiovascular events was 2.2 times higher, while for CRP the risk was 4.4 times higher. Pradhan et al examined the presence of type 2 diabetes in the Women's Health Study population at 4-year follow-up and found that the increased risk of developing type 2 diabetes in the highest versus lowest quartile was 2.3 for IL-6 and 4.2 for CRP, after adjustment for BMI.[14] However, a recent analysis of the Atherosclerosis Risk in Communities (ARIC) study showed that IL-6 was a better predictor of type 2 diabetes than CRP or sialic acid even after adjustment for BMI and baseline fasting insulin and glucose.[16]

In spite of considerable evidence linking CRP to disease outcomes,[13-15,17,29,30] this analysis suggests that CRP may not be the most useful marker of the inflammatory response with a DR significantly worse than a number of other inflammatory markers. It is therefore plausible that previous reported associations may underestimate the importance of inflammation in the pathogenesis of many diseases. The DR analysis is a useful tool allowing relationships to be adjusted for within-individual differences in measures. However, use of the DR is limited to the population in which it was derived. The DR therefore needs to be considered as an integral part of the planning and design of future study analysis.

### *Conclusion*

This study suggests that a single measurement of serum sialic acid may be the most useful estimate of an individual's habitual inflammatory status. It has the highest DR of the tested acute phase markers and, although the DR for the cytokine IL-6 is greater, the methodological difficulties of measuring IL-6 make sialic acid a more appropriate choice for large-scale epidemiological studies. Where possible, estimates of both sialic acid and IL-6 may enhance the ability to describe the mechanistic relationship between inflammatory status and disease.

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