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# Multiplex assay reliability and long-term intra-individual variation of serologic inflammatory biomarkers

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ABSTRACT

*Background:* Circulating cytokines, chemokines, and soluble cytokine receptors can serve as biomarkers of inflammation and immune dysregulation. Good reliability of multiplex platforms, which allow for simultaneous, comprehensive biomarker assessment, is critical for their utility in epidemiologic studies. We examined the reliability of the Meso-Scale Discovery (MSD) platform to simultaneously quantitate 15 cytokines and chemokines and the Luminex platform (R&D Systems) to quantitate 5 soluble receptors and 2 chemokines and cytokines and evaluated long-term within-person correlation of these biomarkers.
*Methods:* The detectability and reliability of these assay systems were assessed using the same external controls across plates and archived sera from 250 HIV$^-$ men in the Multicenter AIDS Cohort Study. Using up to four visits per person from 1984 to 2009, age-adjusted intraclass correlation coefficients (ICC) of biomarkers with >80% detectability (CCL11, CXCL8, CXCL10, CCL2, CCL4, CCL13, CCL17, CXCL13, IL-10, IL-12p70, IL-6, TNF-α, BAFF, sCD14, sCD27, sgp130, sIL-2Rα, and sTNF-R2) were obtained using linear mixed models.
*Results:* Most biomarkers were detectable in 80% of control samples; IFN-γ, GM-CSF, and IL-2 were undetectable in >20% of samples. Among the HIV-uninfected men, most biomarkers showed fair to strong within-person correlation (ICC > 0.40) up to 15 years. The ICC for CXCL8 was good in the short term but decreased with increasing time between visits, becoming lower (ICC < 0.40) after 8 years.
*Conclusions:* These multiplexed assays showed acceptable reliability for use in epidemiologic research, despite some technical variability and limitations in cytokine quantitation. Most biomarkers displayed moderate-to-excellent intra-individual variability over the long term, suggesting their utility in prospective studies investigating etiologic associations with diverse chronic conditions.


## 1. Introduction

Inflammation and immune activation are associated with chronic health conditions, including malignancies [1,2], cardiovascular disease [3], kidney and liver dysfunction [4,5], and AIDS [6]. Chronic inflammation is characterized by persistent activation of the innate and adaptive immune systems. Circulating serologic biomarkers, such as chemokines, cytokines, soluble cytokine receptors, and acute phase proteins are commonly used in epidemiologic

*Abbreviations:* BAFF, B-cell activating factor; BMI, body mass index; CI, confidence interval; CCL11, C-C motif ligand 11; CV, coefficient of variation; CXCL8, C-X-C motif ligand 8; GM-CSF, granulocytemacrophage colony-stimulating factor; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICC, intraclass correlation coefficient; IFN, interferon; IL, interleukin; IQR, interquartile range; LLOD, lower limit of detection; sIL-2Rα, soluble IL-2 receptor alpha; MACS, Multicenter AIDS Cohort Study; sCD14, soluble cluster of differentiation 14; sgp130, soluble glycoprotein 130; TNF-α, tumor necrosis factor-alpha; sTNF-R2, soluble tumor necrosis factor-receptor 2.

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studies to understand underlying inflammatory mechanisms associated with disease risk and progression. Most prior work has focused on single or small numbers of biomarkers, such as TNF-α, IL-6, and C-reactive protein (CRP). While informative, this approach offers an incomplete picture of the complex inflammatory response comprised of multiple interacting circulating mediators. Multiplex technologies permit concurrent testing of large numbers of analytes using minimal sample volume, allowing for rapid, cost-effective quantitation of a more comprehensive panel of biomarkers. While it is important to capitalize on these emerging methods, formal assessment of assay reliability is warranted. Prior studies of multiplex reliability have been restricted by small sample sizes or a limited number of biomarkers [7,8].

Furthermore, studies often use a single blood sample to quantitate circulating concentrations of inflammatory biomarkers and characterize a participant's risk, assuming that a single measurement represents the individual's long-term state of inflammation. High within-person variability can result in measurement error, biasing risk estimates towards the null and attenuating the likelihood of identifying valid exposure-disease associations. The intraclass correlation coefficient (ICC) assesses inter-individual (between people) variability relative to total variability (between and within individuals) and provides a measure of the extent to which a biomarker tracks within a person over time. Low ICCs necessitate multiple measurements over time to more accurately capture the inflammatory state, while low variability within a person, or high tracking, improves the precision of estimates from longitudinal studies [9]. Biomarkers exhibiting constant within-person correlation over time may also suggest a lack of immunological response to transient or acute exposures, potentially offering an important insight into the relationship between biomarkers and disease [10]. Previous studies of within-person biomarker variability have been limited by small sample sizes, a narrow range of biomarkers, or relatively short periods of time between biomarker measurements (⩽5 years) [11–20].

This study had two aims: (1) to determine the detectability and reliability of the Meso-Scale Discovery (MSD) and Luminex platforms; and (2) to evaluate the long-term within-person correlation of 22 biomarkers of inflammation in a well-characterized, long-standing prospective cohort study. Results from this study will aid in designing future epidemiologic studies on the role of inflammatory biomarkers in disease etiology.

## 2. Materials and methods

### 2.1. Study design and population

This analysis was conducted within the Multicenter AIDS Cohort Study (MACS), a prospective cohort study of men who sex with men (MSM) enrolled at four U.S. locations (Baltimore/Washington D.C., Chicago, Los Angeles, and Pittsburgh) to examine the natural and treated histories of HIV-1 infection. Since 1984, 6972 participants have been enrolled: 4954 in 1984–1985, 668 in 1987–1991, and 1350 in 2001–2003. Institutional review boards at each center approved the MACS protocols and informed consent was obtained from all participants. Descriptions of the MACS protocol have been published previously [21,22]. Study highlights, including data collection forms, may be found at https://statepi.jhsph.edu/macs/macs.html. Briefly, study participants are evaluated every six months with standardized interviews, physical examinations, and laboratory analysis of collected blood. Serum, plasma, and peripheral blood mononuclear cells are frozen and stored in local and national repositories. Serum samples used in this study were previously unthawed.

Control plasma samples isolated from an HIV-uninfected donor and a HIV-infected donor during a single blood draw were aliquoted and frozen. These external control plasma samples were obtained from Thomas N. Denny at Duke Human Vaccine Institute, Immunology and Virology Quality Assessment Center. Duke University IRB approvals were in place for these activities. To evaluate the reliability of the MSD platform, previously unthawed control plasma samples were run in duplicate on each plate over the course of the study.

To assess the ICCs, the concentrations of 22 inflammatory biomarkers were measured in serum samples from 250 HIV-uninfected men. Four study visits per individual were selected to represent the age and race distributions of the underlying cohort population from 1984 to 2009. All HIV-uninfected MACS men who were hepatitis C (HCV)-infected were included, to provide sufficient numbers for comparative analyses. All longitudinal specimens per individual were run on the same plate.

### 2.2. Laboratory methods

#### 2.2.1. MSD platform

Serum concentrations of 9 cytokines and 7 chemokines were determined using the Meso-Scale Discovery (MSD) platform (Meso-Scale Diagnostics, LLC, Rockville, MD). The MSD system is an electrochemiluminesence-based, 96-well format solid-phase multiplex assay. Two separate kits, the Human Proinflammatory 9-Plex Ultra-Sensitive kit and Human Chemokine 7-Plex Ultra-Sensitive kit, were used to determine concentrations of IL-1β, IL-2, IL-6, IL-10, IL-12p70, IFN-γ, GMCSF, TNF-α, and CCL11, CXCL10, CCL2, CCL13, CCL4, and CCL17, respectively. CXCL8 was included in both kits. MSD assays were performed according to the manufacturer's instructions. All MSD testing was conducted in a single laboratory by a single technician at the Johns Hopkins Bloomberg School of Public Health.

Cytokine limits of detection ranged from 0.8 to 1.2 pg/ml, while those for chemokines ranged from 39 to 158 pg/ml. Standard curves were done on each plate in duplicate. The lower limit of detection (LLOD) for each plate-specific analyte was the concentration 2.5 standard deviations above the background.

#### 2.2.2. Luminex platform

Serum concentrations of the soluble receptors were determined using the multiplexed Luminex xMAP system (Fluorokine® MAP) using assays produced by R & D systems (Minneapolis, MN) following the manufacturer's instructions, and a Bio-Plex 200 Luminex instrument and Bio-Plex software (Bio-Rad, Hercules, CA). Concentrations of four soluble receptors (sCD14, sgp130, sIL-2Rα, sTNF-R2), plus a cytokine (BAFF) and the chemokine CXCL13, were measured in a single panel (Human Biomarker Custom Premix Kit A). All testing for these markers was conducted in a single laboratory at the University of California, Los Angeles. One external serum control from a normal donor (no spiked values) was run in duplicate on each assay. This control sample was from a single blood draw that was aliquoted multiply and stored at −80 °C. For each plate tested, a biomarker- and plate-specific LLOD was defined. All the samples tested in the multiplex Kit A had concentrations above the lowest standard of the standard curve; therefore, the LLOD was defined as the observed concentration of the lowest standard.

### 2.3. Statistical methods

The detectability and reliability of each platform were calculated using the external control samples. Detectability was defined as the proportion of samples above the assay's LLOD. The plate-specific intra-assay coefficient of variation (CV) for each