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Temporal stability of serum concentrations of cytokines and soluble receptors measured across two years in low-risk HIV seronegative men

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Abstract

Background—Prospective cohort studies often quantify serum immune biomarkers at a single time point to determine risk of cancer and other chronic diseases that develop years later. Estimates of the within-person temporal stability of serum markers partly assess the utility of

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single biomarker measurements, and may have important implications for the design of prospective studies of chronic disease risk.

Methods—Using archived sera collected from 200 HIV-seronegative men at three visits spaced over approximately two years, concentrations of 14 biomarkers (ApoA1, sCD14, sgp130, sIL-6R, sIL-2Rα, sTNFR2, BAFF/BLyS, CXCL13, IFN-γ, IL-1β, IL-6, IL-8, IL-10, TNF-α) were measured in a single laboratory. Age-and ethnicity-adjusted intraclass correlation coefficients (ICC) were calculated for each biomarker, and mixed linear regression models were utilized to examine the influence of age, ethnicity, season, and study site on biomarker concentrations.

Results—Across all three study visits, most biomarkers had ICC values indicating fair to excellent within-person stability. ApoA1 (ICC=0.88)and TNF-α (ICC=0.87) showed the greatest stability; the ICC for IL-8 (ICC=0.33) was remarkably less stable. The ICCs were similar when calculated between pairs of consecutive visits. The covariables did not influence biomarker levels or their temporal stability. All biomarkers showed moderate to strong pairwise correlations across visits.

Conclusions—Serum concentrations of most evaluated immune biomarkers displayed acceptable to excellent within-person temporal reliability over a 2-year period. Further investigation may be required to clarify the stability of IL-8.

Impact—These findings lend support to using these serologic immune biomarkers in prospective studies investigating associations with chronic diseases.

Introduction

The role of immune dysregulation in the etiology of chronic diseases has been a topic of much recent research, including studies on the etiology of hematological malignancies (reviewed in (1)), solid tumors (2-6), and non-malignant conditions (7-9). Studies of immune marker-disease associations are often limited to a single measurement of each biomarker per study participant, using blood samples that may have been collected years before a target disease is diagnosed. It is important to understand the within-person temporal stability of the immunologic biomarkers of interest so that the potential misclassification of participants' immune status is adequately considered in the interpretation of the etiologic data.

Several studies have provided evidence of reasonable within-person temporal stability, as characterized by the intraclass correlation coefficient (ICC), for panels of human cytokines and soluble cytokine receptors (10-16). However, existing studies were limited by small sample sizes, or covered relatively short periods of time. In addition, those studies did not include some inflammatory, cytokine signaling, or chemoattractant molecules that may be informative markers of chronic disease risk. We therefore undertook the present study to assess the within-person temporal reproducibility of four molecules with no previous reports on temporal stability (ApoA1, soluble gp130 [sgp130], BAFF/BLyS, and CXCL13), as well as 10 previously evaluated immune markers in longitudinal serum samples collected over two years from 200 HIV-seronegative men in the Multicenter AIDS Cohort Study (MACS). The molecules included in this study are known to be associated with inflammatory responses and immune system activation (1). As such, this study is the largest to date to investigate the within-person temporal stability of a comprehensive panel of cytokines and soluble receptor molecules of potential interest to etiologic studies.

Materials and Methods

Study Population

The present analysis was conducted within the Multicenter AIDS Cohort Study (MACS), a prospective study of 6,972 homosexual and bisexual men enrolled at four US locations (Baltimore/Washington DC, Chicago, Los Angeles, Pittsburgh) to investigate the natural and treated histories of HIV-1 infection (17, 18). The present study population consists of 200 HIV-seronegative men considered to be at low risk of seroconversion based on self-reported sexual behaviors, who formed the control group for a study of factors associated with resistance to HIV infection (19). Most of the study population joined during the first enrollment period in the MACS (1984-1985);all 200 subjects gave blood samples at three different study visits spaced approximately one year apart between 1984 and 1987. The MACS was approved by the Institutional Review Board (IRB) at each study site, and each participant provided informed consent. The protocol for the present analysis was approved by the IRB of Brigham and Women's Hospital.

Serological tests

The present study was performed in blood samples collected during routine follow-up visits by cohort members to one of the four MACS study sites. The specimens were kept at room temperature before processing, with an estimated mean time of 6 hours between blood draw and freezing (at -70°C) (20). Each of the four sites followed the same shared protocol to maximize the consistency with which blood samples were collected and processed. Three frozen, archived serum samples from each of the 200 present study participants (i.e., a total of 600 samples) were obtained from the MACS sample repository in 2010-11, and tested to determine the concentrations of 14 serum immune markers. Assays were performed using multiplexed (Luminex platform) assay kits (Fluorokine® MAP, R & D Systems, Minneapolis, MN) according to manufacturer directions, and a Bio-Plex 200 Luminex instrument and Bio-Plex analysis software (Bio-Rad, Hercules, CA) in a single laboratory. Levels of five soluble receptors, including soluble CD14 (sCD14), soluble gp130 (sgp130), soluble IL-6 receptor (sIL-6R), soluble IL-2 receptor- (sIL-2R ; also known as sCD25), and soluble TNF receptor-2 (sTNFR2), plus a cytokine known as B cell activating factor or B Lymphocyte Stimulator (BAFF/BLyS), and the chemokine CXCL13 (also called B-lymphocyte Chemoattractant/B Cell-attracting Chemokine 1; BLC/BCA1), were measured in one panel (Human Biomarker Custom Premix Kit A); Apolipoprotein A1 (ApoA1), a component of high density lipoprotein (HDL) that has anti-inflammatory properties, was measured separately in a single-plex assay, due to the much greater sample dilution required for this assay. The concentrations of six additional human cytokines, including interferon (IFN)- , interleukin (IL)-1 , IL-6, IL-8 (also called CXCL8), IL-10, and tumor necrosis factor (TNF)- , were determined in a second high-sensitivity panel (Human Inflammation Multiplex Kit). To monitor assay performance, duplicate quality control (QC) serum samples from a healthy adult donor were tested on each assay plate. Intra-assay coefficients of variation (CV) were computed from the QC duplicates tested within each plate, and inter-assay CVs were computed from the QC sample concentrations observed across all the plates (i.e., across each within-plate mean QC sample concentration). For the Human Biomarker Kit A, the mean intra-assay CV ranged from 5.4% (sIL-2R) to 8.2% (sCD14), and the inter-assay CV ranged from 10.5% (sTNF-R2) to 19.5% (CXCL13). For the ApoA1 assay, the mean intra-assay CV was 6.7% and the inter-assay CV was 41.0%. For the Human Inflammation Kit, the mean intra-assay CV ranged from 5.5% (IL-8) to 7.4% (TNF), and the inter-assay CV ranged from 11.3% (IL-8) to 15.9% (TNF).

For each plate of serum samples tested, a biomarker-and plate-specific lower limit of detection (LLD) was defined. In the multiplex Kit A and ApoA1 assays, all samples had

concentrations above the lowest standard of the standard curve, and thus the LLD was defined as the observed concentration of the lowest standard. For the high-sensitivity cytokine multiplex assays (IFN- , IL-1 , IL-6, IL-8, IL-10, TNF-) the LLD was defined as the lowest value of either sample or standard on each plate. A recent comparison of high-sensitivity multiplex cytokine assays indicated that assay sensitivity is effectively limited to concentrations above 0.1 pg/mL (21). For this reason, in the present analysis we considered all extrapolated concentrations of 0.1 pg/mL as below the LLD. All observations deemed undetectable were assigned a value equal to one-half the plate-specific LLD and retained in the analysis. Four observations (0.7%; three measurements of sIL6-R and one of IL-8) were recorded at levels above the upper limit of detection due to rounding and were set to values equal to the corresponding plate-and biomarker-specific upper limits of detection.

Statistical Analysis

Biomarker levels were natural log-transformed to improve normality. The within-and between-person variances were estimated for each biomarker using mixed linear regression models, which can incorporate both the "fixed" effects of the potential confounding variables we examined and the subject or "random" effects and the correlation among repeat measures from any given individual in the longitudinal data (22, 23). We then computed a corresponding ICC for each biomarker and 95% confidence intervals (CI) to evaluate precision (24). The ICC indicates the proportion of the total variability that is due to between-subject variability, relative to the proportion that is from within-person variability, and is commonly used in studies of biomarker reproducibility (25). A larger ICC suggests that the given biomarker is relatively stable within a given person over the time period covered by the repeated measurements, in comparison to the variability observed across the different individuals in the study. For the main analysis, ICCs were calculated using all three repeat measurements. In a sensitivity analysis, we also computed an ICC for each pair of consecutive study visits (i.e., visits 1 and 2, and visits 2 and 3). We performed models for each biomarker without adjustment for covariables as well as multivariable models to assess potential confounding of the ICCs by age, ethnicity, season of blood draw and MACS study site. For comparison with the ICCs, we computed age-adjusted Spearman partial correlation coefficients for each pair of repeated measurements of each serologic marker. Spearman correlations do not distinguish within-person from between-person variability or account for the inherent relatedness of the repeated measures from the same individual, but rather assess the monotonic relationship between two data items, such as repeat measurements of biomarkers at consecutive study visits (26). In addition to the Spearman correlations computed across pairs of study visits, we also calculated age-adjusted partial Spearman correlation coefficients to assess the cross-sectional correlation between each possible pair of biomarkers measured at the same visit.

We next utilized mixed linear regression models to examine the association of age at blood draw, season of blood draw, ethnicity, and MACS study site with the biomarker levels. All models included a random effect for subject to account for the within-person correlations and used visit number (1, 2, 3) as the time scale.

All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Statistical hypothesis tests were two-tailed and assumed an alpha error of 0.05.

Results

Among the 200 men included in our analysis, the median age at visit 1 was 34.5 years (range 21.6-54.6 years; Table 1). The population was largely Caucasian (96%), and averaged approximately one year (range 0.4-1.5 years) between the study visits from which serum samples were obtained for this analysis.

Samples considered to be undetectable, i.e., with concentrations below the plate-specific LLD or with calculated concentrations 0.1 pg/mL, were recorded for five cytokines (Table 2). IL-6, IL-10, and TNF- were undetectable in only a small number of observations (<11%); in contrast, IFN- and IL-1 had higher percentages of undetectable samples (31.7% and 38.5%, respectively).

In analyses of the repeated biomarker measurements from all three study visits, 13 of the biomarkers achieved an ICC >0.40 (Table 3). Among those markers with stronger ICCs, the within-person temporal stability appeared greatest for ApoA1, which had an ICC of 0.88 (95% CI: 0.85, 0.90), and TNF-, which had an ICC of 0.87 (95% CI: 0.84, 0.90). The ICC was also very strong for several other soluble receptors and moderately strong for several cytokines. However, one cytokine, IL-8, had a relatively low age-and ethnicity-adjusted ICC of 0.33 (95% CI: 0.24, 0.42), suggesting poor temporal reproducibility (26). Adjustment for age and ethnicity did not notably change the ICCs and 95% CI limits for any immune marker (Table 3). Similarly, additional adjustment for season of blood draw or MACS study site did not affect the ICCs or 95% CIs for any biomarker (data not shown). The ICCs calculated for each biomarker between pairs of consecutive study visits were very similar to the ICCs calculated across all three visits (Supplementary Table 1), with ApoA1 and TNF-having the greatest within-person temporal stability.

In general, the biomarkers that had stronger ICCs also had stronger correlations across pairs of study visits (Supplementary Table 2), whereas the correlations across visits were less strong for cytokines IL-1, IL-8, and IL-6. When correlations were calculated between different biomarkers measured at the same visit (Supplementary Tables 3-5), we observed that the levels of many of the soluble receptors showed moderately strong correlations with each other (r 0.30). The most consistently strong correlation across all the immune markers was seen between sIL2-R and sTNF-R2, with a correlation coefficient of approximately 0.6 at each visit. CXCL13, a chemokine and B cell attractant, showed much weaker correlations with the soluble receptors at any visit. The soluble receptor concentrations were not strongly correlated with levels of any cytokines.

In addition to the examination of temporal reproducibility and between-marker pairwise correlation, we performed mixed linear regression analysis to evaluate the association between selected covariables and the biomarker concentrations. In those models, the geometric mean biomarker concentrations did not vary systematically between Caucasian and non-Caucasian study participants, by MACS study site, or by season of blood draw (data not shown).

Discussion

The present study assessed the within-person temporal stability of 10 previously evaluated immune markers and four serologic immune markers with no previous reports on temporal stability (ApoA1, sgp130, BAFF/BLyS, and CXCL13) in longitudinal blood samples provided over two years or longer by 200 HIV-seronegative men. This study is the largest to date to investigate the within-person temporal stability of a comprehensive panel of cytokines and soluble receptor molecules. Several previously published reports assessing temporal stability in serum biomarkers have proposed to define adequate within-person temporal stability by an ICC value 0.55 (13, 27), thus suggesting that a single measurement of those biomarkers could be incorporated into a prospective study of disease risk. By that criterion,12 of the 14 biomarkers measured in our multi-marker panel of cytokines and soluble receptor molecules show acceptable to excellent within-person temporal stability over a time period of up to 2.5 years, including the four biomarkers not previously reported (ApoA1, sgp130, BAFF/BLyS, and CXCL13). By other widely used criteria that consider

ICCs 0.40 to be adequate (26, 28), all of the biomarkers in our panel except IL-8 can be considered suitable for use in prospective studies of chronic disease risk. Within-person variation may reduce the observed correlation between two variables (29), such as a biomarker measured at two separate time points, and can limit the statistical power of epidemiologic studies to detect an association of a biomarker measured only once with disease risk. Thus, the more stringent criterion defining adequate within-person variability by an ICC 0.55 may be best in studies with limited statistical power. When available, ICCs from reproducibility studies can be applied to statistical methods for measurement error correction. In the context of evaluating assay reliability, such methods better estimate the "true" disease relative risk associated with the biomarker of interest given the observed relative risk and reported ICC, and thereby account for the "error" in exposure classification and its influence on the observed biomarker-disease associations (28, 30, 31). Statistical correction can also be performed using ICCs from within-person temporal stability studies such as the present analysis. Those statistical corrections can be informative for interpreting observed biomarker-disease associations based on a single biomarker measurement when the ICCs suggest relatively poor within-person temporal stability. Also, in the absence of longitudinal biomarker measurements, a high ICC gives credence to the persistence of relative rankings of exposure among participants, at least over a short time period.

We observed lower within-person temporal stability in measurements of IL-8, a proinflammatory cytokine, which is consistent with some prior publications (13, 15). However, other publications have shown reasonable stability for IL-8 (11, 16) including analyses using high-sensitivity assays.(14) It may be that a subset of the men in the present analysis experienced periodic exposure to immune-activating stimuli, which in turn led to acute spikes in IL-8 secretion. However, we would have expected other proinflammatory cytokines (e.g. IL-1 and IL-6) to also be affected by such transient immune activation, and we did not detect similarly poor within-person temporal stability in the other proinflammatory cytokines.

Our study has several strengths. As previously noted, this analysis is the largest to date to analyze the within-person temporal stability of multiple serologic biomarkers of host immune status. The large sample size reduced the variance of our estimates, and allowed us to examine the possible influence of covariates on biomarker variability with precision. The precision of our ICC calculations is illustrated by the narrow range of the corresponding 95% confidence intervals. Furthermore, our analysis investigated four immune markers not previously analyzed for temporal stability (ApoA1, sgp130, BAFF/BLyS, CXCL13), and demonstrated good stability across time for these biomarkers. Our findings of good temporal stability of TNF- , sTNFR2, and IL-10 agree with the literature (10, 13-16), as do our findings for sIL-2R and IFN- in studies using high-sensitivity assays (14). Most published reports suggest greater temporal stability for IL-6 than we observed in our study (11,13-15), although others are concordant with our findings (10,16), which generally indicate good within-person stability. Estimates in the literature for stability of sIL-6R have been varied, although generally strong (13, 14), consistent with the present findings. We had the additional benefit of comparing biomarker levels measured in samples collected at three separate visits over a timespan of more than two years, representing one of the longer studies of biomarker stability. To our knowledge, only the study by Hofmann et al. (15), which included repeat samples from 28 participants in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO), covered a markedly longer period of calendar time (5 years). Among the cytokine markers that were included in both the PLCO and the present study, the ICCs for IL-10 and TNF- were similar in the two populations, while those for IL-6 and IL-8 were greater in the PLCO. IL-1 was generally undetectable in the PLCO, and an ICC not reported (15).

The present study also has limitations that warrant consideration. First, although we were able to assess the stability of biomarker levels over more than two years, we cannot confirm the stability of these biomarkers over longer periods of time. Investigators who conduct prospective studies of the associations between levels of the immune biomarkers in our analysis and chronic disease endpoints utilizing blood samples collected many years before disease development can derive some reassurance from our study. The use of measurement error correction methods could be warranted in such studies, where within-person variability and the related loss of statistical power could be potential explanations for null findings. Also, the study population was unique in that it was composed of young men who have sex with men, and did not contain women, children or older persons.

In conclusion, the findings from our analysis suggest that levels of the majority of serological biomarkers of immune function assessed in our study (ApoA1, sCD14, sgp130, sIL-6R, sIL-2R , sTNFR2, BAFF/BLyS, CXCL13, IFN- , IL-1 , IL-6, IL-10, and TNF-) are relatively stable within-person over two or more years, whereas levels of IL-8 appeared to be less stable. Our findings can be incorporated into statistical corrections to diminish the influence of within-person temporal stability on observed immune marker-disease associations, especially for the cytokines and soluble receptors that demonstrated only fair to moderate stability. With continued advances in immunologic and serologic technologies available for high-throughput use in epidemiological studies, additional studies will continue to be necessary to assess the within-person temporal stability of novel markers. Cohorts that have stored longitudinal samples over longer time intervals would contribute valuable information to the literature by performing assessments of within-person temporal stability of immune biomarkers, given their potential application to etiologic studies of diverse chronic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Descriptive characteristics of 200 HIV seronegative male MACS participants at 3 different study visits

Variable	N (%)	Median	Range
Overall Age, years			
Visit 1	200	34.5	21.6 -54.6
Visit 2	200	35.5	22.5 -55.6
Visit 3	200	36.5	23.7 -56.7
Ethnicity[a]			
White	192 (96%)		
Non-white	8 (4%)		
Dates of visits			
Visit 1	200	3/1985	4/1984 -9/1985
Visit 2	200	2/1986	5/1985 -9/1986
Visit 3	200	2/1987	11/1985 -6/1987
Time between visits, years			
Visits 1 and 2	200	0.98	0.40 -1.52
Visits 2 and 3	200	0.96	0.40 -1.50
Visits 1 and 3	200	1.94	1.02 -2.45

[a]Self-reported ethnicity or race

Table 2

Serum concentrations (pg/mL) of 14 immune biomarkers measured in 200 HIV seronegative men at 3 different study visits

Biomarker	Percent observations within limits of detection	Biomarker concentrations (pg/mL)			
		Minimum[a]	Geometric Mean[b]	Median	Maximum
ApoA1	100%	0.43×10^9	1.27×10^9	1.15×10^9	4.57×10^9
sCD14	100%	0.49×10^6	2.02×10^6	2.05×10^6	3.89×10^6
sgp130	100%	0.74×10^5	2.35×10^5	2.40×10^5	4.19×10^5
sIL-6R	100%	1.42×10^4	4.65×10^4	4.74×10^4	8.09×10^4
sIL-2R	100%	584	1360	1371	3469
sTNF-R2	100%	658	1950	1995	5719
BAFF/BLyS	100%	844	2018	2025	4695
CXCL13[c]	100%	34	299	302	6507
IFN-	68.3%	BLD	0.2	0.4	4.4
IL-1	61.5%	BLD	0.2	0.2	16.2
IL-6	89.3%	BLD	0.9	1.3	23.7
IL-8	100%	1.2	11.6	10.3	6406
IL-10	94.2%	BLD	0.6	0.7	10.8
TNF-	96.2%	BLD	4.7	6.0	22.6

[a] BLD = lowest values were below the lower limit of detection

[b] Calculated using natural log-transformed values, back-transformed to original scale

[c] One observation with an implausible value for CXCL13 was excluded only from analyses of CXCL13 (Total N = 599)

Table 3

Intraclass Correlation Coefficients (ICC) and 95% Confidence Intervals (CI) for 14 immune biomarkers measured in 200 HIV seronegative men, calculated over 3 study visits

	Unadjusted		Adjusted for age and ethnicity[b]	
Biomarker[a]	ICC	95% CI	ICC	95% CI
ApoA1	0.88	(0.85, 0.90)	0.88	(0.85, 0.90)
sCD14	0.57	(0.49, 0.64)	0.56	(0.49, 0.64)
sgp130	0.70	(0.64, 0.75)	0.69	(0.63, 0.75)
sIL-6R	0.82	(0.78, 0.86)	0.82	(0.78, 0.85)
sIL-2R	0.79	(0.75, 0.83)	0.79	(0.75, 0.83)
sTNF-R2	0.73	(0.68, 0.78)	0.73	(0.68, 0.78)
BAFF/BLyS	0.70	(0.64, 0.76)	0.70	(0.64, 0.76)
CXCL13[c]	0.70	(0.65, 0.76)	0.70	(0.64, 0.76)
IFN-	0.55	(0.47, 0.62)	0.55	(0.47, 0.62)
IL-1	0.49	(0.41, 0.57)	0.48	(0.40, 0.56)
IL-6	0.55	(0.47, 0.62)	0.54	(0.47, 0.62)
IL-8	0.34	(0.25, 0.43)	0.33	(0.24, 0.42)
IL-10	0.70	(0.64, 0.75)	0.70	(0.63, 0.75)
TNF-	0.87	(0.84, 0.90)	0.87	(0.84, 0.90)

[a] Undetectable values were assigned a value equal to one-half the plate-specific lower limit of detection

[b] Age at visit was modeled continuously; ethnicity or race was self-reported

[c] One observation with an implausible value for CXCL13 was excluded only from analyses of CXCL13 (Total N = 599)