Biomarkers in Behavioral Medicine

Risana N. Chowdhury
College of Behavioral and Community Sciences
University of South Florida-Tampa

Introduction

When incorporating biomarkers into behavioral medicine research, specimen storage and handling best practices can help ensure the accuracy of results. Bioarchived specimens can also be used in research, however protein degradation can be of concern.

This study evaluates the stability of 16 serum biomarkers from 2(baseline) to 50 freeze-thaw cycles (FTCs).

This study aims to answer two questions:
1) Are serum biomarker levels statistically equivalent from baseline to 25 and baseline to 50 FTCs?
2) Are patterns retained across FTCs for the 16 serum biomarkers of interest?

Table 1: Selected physiological and disease-related biomarker functions

Biomarker	Function
C-reactive protein (CRP)	Widely assessed in biological studies [1]; marker of hepatic origin involved in acute and chronic inflammation [2].
Interleukin 1a (IL-1a)	Associated with cardiovascular disease, colon cancer and neuro-inflammation and autoimmune disease [3].
Interleukin 4 (IL-4)	Involved in differentiation of naïve B and T cells. Part of type II immune response against helminths and toxins [4].
Interleukin 6 (IL-6)	Activates immune responses after inflammation or injury [5]. Involved in autoimmune and respiratory disease and chronic inflammation; often evaluated alongside CRP [6].
Interleukin 8 (IL-8)	Attracts and activates neutrophils in inflammatory regions [5]. Increased expression is associated with many cancers, diseases of inflammation (e.g., rheumatoid arthritis) and infection [7].
Interleukin 10 (IL-10)	Involved in a broad range of anti-inflammatory mechanisms in disease and homeostasis [5].
Monocyte Chemoattractant Protein 1 (MCP-1)	Activates macrophages and other immune cells, recruits inflammatory cells after inflammation or injury [5]. Secreted by different cell types; associated with many conditions including rheumatoid arthritis, atherosclerosis and multiple sclerosis [8].
Monocyte Chemoattractant Protein 2 (MCP-2)	Chemotactic for and activates monocytes and other immune cells [5]. Involved in the induction of labor during childbirth, breast cancer metastasis, suppression of HIV-1 [9]
Interferon Gamma (IFN-γ)	Induces MHC class-II; related to anti-viral, antibacterial, anti-protozoan, immunoregulatory, and anti-tumor effects [5].
IFN-γ-Induced Protein 10 (IP-10)	Secreted in response to IFN-γ; regulates immune responses of T cells and other immune cells [5].
CCL5 (RANTES)	Recruit immune cells, regulates immune responses, related to maintenance of the inflammatory state [5].
CCL11 (Eotaxin-1)	Recruit eosinophils to inflammatory regions, responds to sites of allergic reactions [5].
CCL17 (TARC)	Induces chemotaxis of T-cells [5].
Growth Related Oncogene Alpha (GRO-α)	Associated with malignant melanoma growth, atherosclerosis, chronic obstructive pulmonary disease and angiogenesis [10].
Tumor Necrosis Factor Alpha (TNF-α)	Associated with chronic inflammation and autoimmune disease [11].
CCL1 (I-309)	Associated with cancer cell growth [12]; elevated in active tuberculosis [13].

Materials and Methods

- Sera were collected from 20 healthy volunteers (age >18 years) in the chemistry lab at United Health Services (UHS)-Wilson Memorial Hospital, Johnson City, NY, and transferred into freezer-safe storage vials labeled with an ID number and frozen at -80ºC until all samples were collected within two days.
- Informed consent was obtained from all participants before specimen collection and only information regarding age, sex, and underlying health conditions were recorded; specimens were deidentified. This protocol was approved by the Institutional Review Boards at Binghamton University (SUNY) and UHS
- Specimens underwent consecutive, daily freezing and 1-hour thawing at room temperature (23ºC) for 50 days.
- 300uL aliquots taken at FTC 2(baseline) [lee] and every five FTC.
- Aliquots taken at baseline(2 FTC), 25, and 50 FTC were evaluated.
- Biomarkers evaluated were CRP, IL-1α, 4, 6, 8, 10, MCP-1, MCP-2, eotaxin-1, TARC, RANTES, GRO-α, I-309, IFN-γ, IP-10, and TNF-α. 12 of 20 samples were tested for 16 immunoregulators via ELISA, using the Quansys Bioscience 9-plex High Sensitivity Chemokine Kit (eotaxin-1, GRO-α, I-309, IL-8, IP-10, MCP-1, MCP-2, RANTES, TARC), Quansys Bioscience 4-plex High-Sensitivity Cytokine Kit (IL-4, IL-6, IL-10, IFN-γ), and Quansys Bioscience 4-plex Cytokine Kit (IL-1α, IL-4, IL-6, TNF-α). 20 samples were tested for CRP via in-house CRP immunoassay [14]. All specimens were tested in duplicate, with calculated means used in data analysis. Raw data below the lower limit of detection (LLOD) or with 30%CV were excluded from analyses.
- Two One-sided tests(TOST) of equivalence and Generalized linear models(GLMs) were used in data analysis. Data was analyzed using Rstudio (v2023.12.1) [15,16,17] and SPSS (V.29.0.1.0) statistical software

Results
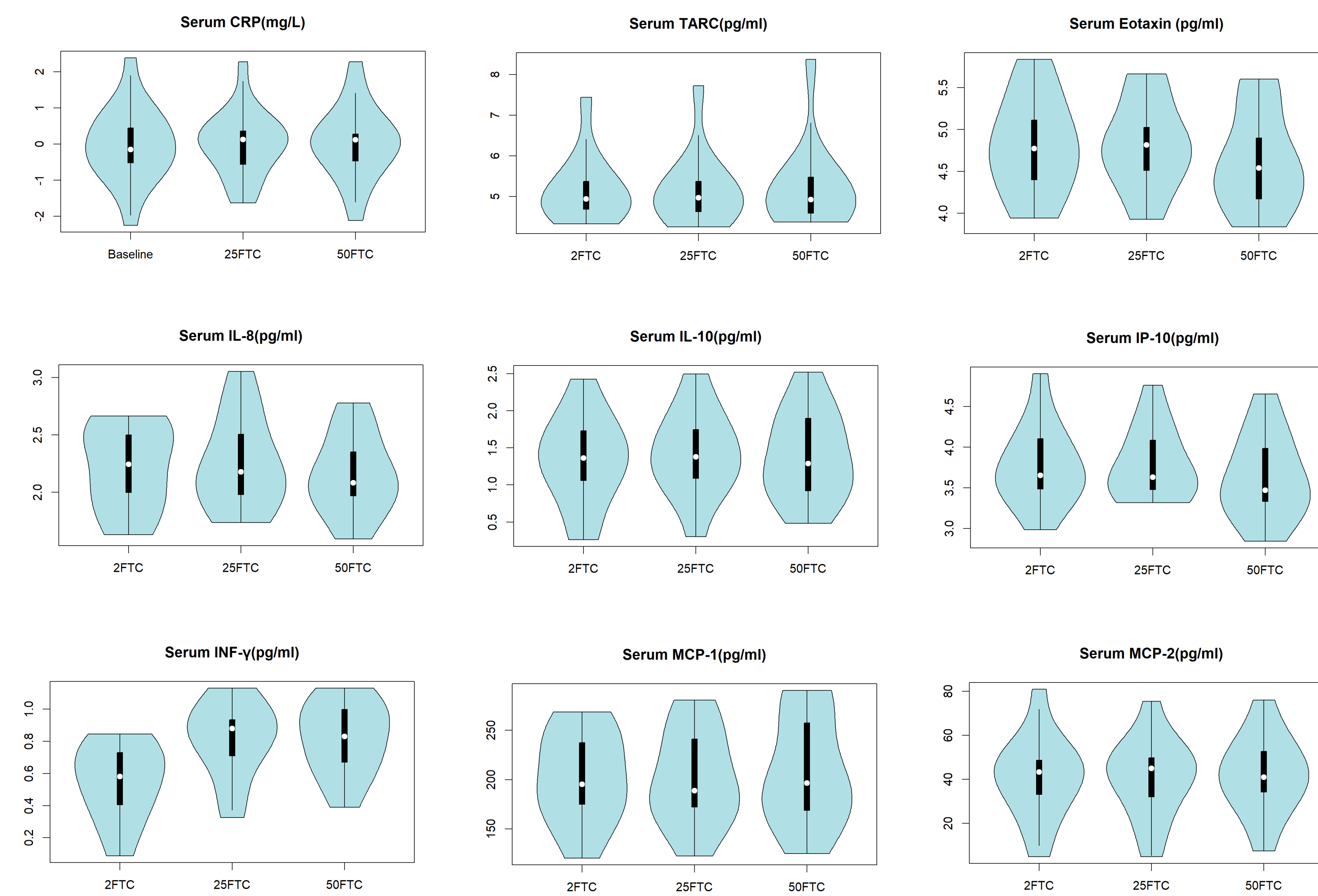
Violin plots depict median values (white dots), inter-quartile ranges and distribution of data [18,19] | x-axes: (log)volume for all biomarkers except IFN-γ, MCP-1 and MCP-2 (actual volumes in pg/mL) | y-axes: 2 (baseline), 25 and 50 freeze-thaw cycles | n=12 for all biomarkers except CRP (n=17) and IFN-γ (n=11).

Table 1: Participant Characteristics

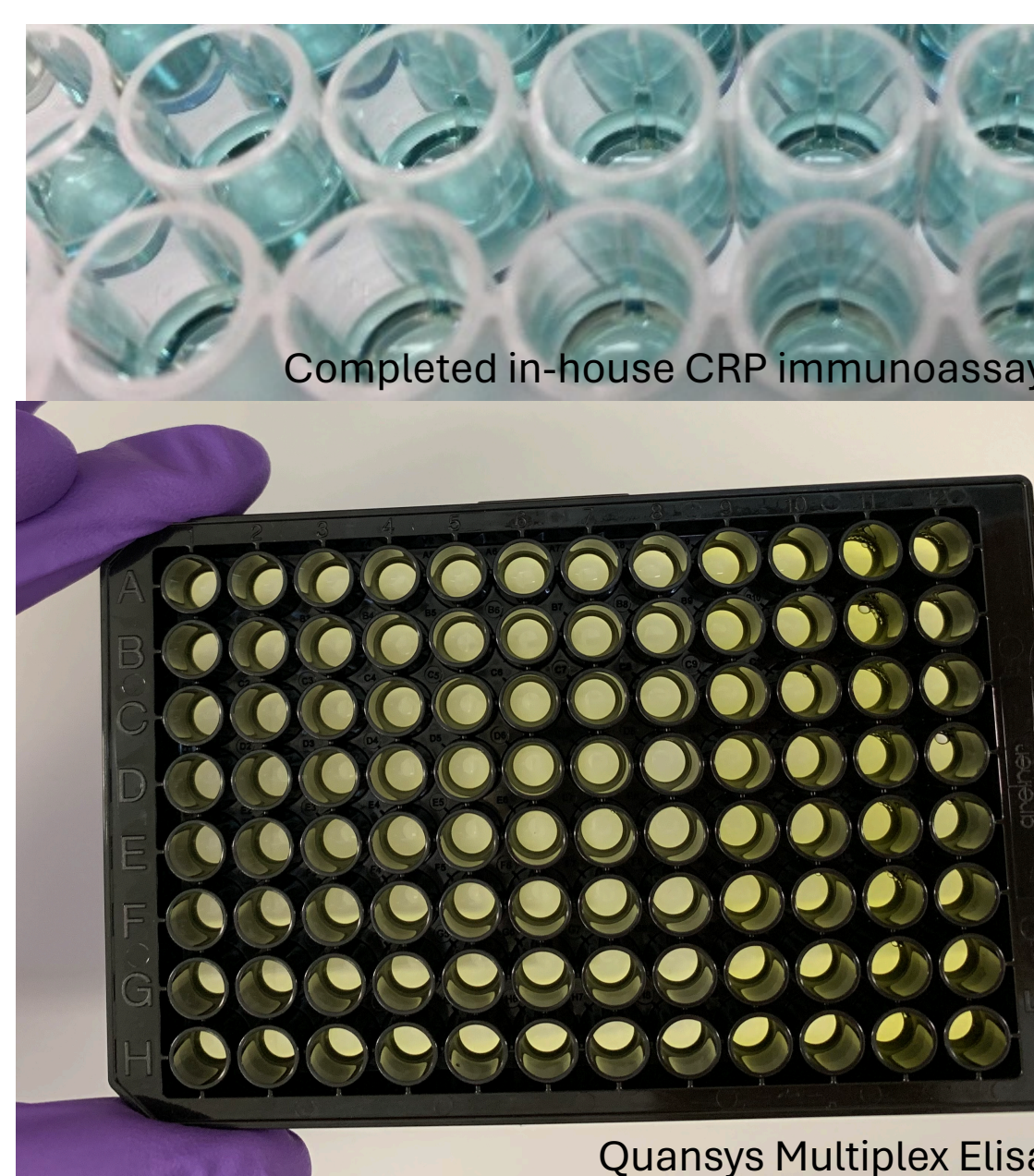| Age Range (years)	Sex
20-30	5 F and 1 M
31-40	2 F and 1 M
41-50	7 F and 0 M
51-60	3 F and 1 M

Completed in-house CRP immunoassay

Quansys Multiplex Elisa

Table 2: Equivalence Tests

Biomarker	Baseline, 25 FTC α [90% CI]	Baseline, 25 FTC Effect Size	Baseline, 50 FTC α [90% CI]	Baseline, 50 FTC Effect Size
CRP§	p<0.001* [-0.57,0.26]	-0.19	p <0.001 [-0.23,0.33]	0.19
IL-8‡	p<0.001 [-0.19,0.12]	-0.04	p <0.001 [-0.1,0.22]	0.06
IL-10‡	p<0.001 [-0.34,0.17]	-0.09	p<0.001 [-0.27, 0.16]	-0.06
IFN-γ†	p <0.001 [-0.3,-0.2]	-0.26	p<0.001 [-0.34,-0.19]	-0.25
IP-10‡	p<0.001 [-0.11,0.06]	-0.03	p<0.001 [0.01,0.29]	0.15
MCP-1†	p=0.46 [-11,11]	0.23	p=0.5 [-23,10]	-6.5
MCP-2†	p=0.32 [-1.99,2.4]	0.18	p=0.54 [-4.37, 2.39]	-0.99
Eotaxin-1‡	p<0.001 [-0.01,0.06]	-0.01	p<0.001 [0.12,0.25]	0.18
TARC§	p=0.2 [-9.3,10]	0.24	p=0.25 [-21,19]	0.07
GROα§	p=0.9 [-33,-17]	1	p=1.0 [-48,-27]	1

Highest p-values and 90% confidence intervals (CI) reported for TOST, *p≤0.05 signifies equivalence | Equivalence bounds (θ) for †Gaussian=0.8,‡Log-adjusted =1.25 and §Non-parametric =0.5 distributions. For nonparametric distributions, rank biserial correlations are reported under *Effect Size* | n=12 for all biomarkers except CRP (n=17) and IFN-γ (n=11).

- IL1a, IL-4, IL-8, TNFα, I-309 results fell below LLOD; RANTES data were >30%CV; these markers were excluded from data analysis.
- Equivalence tests suggest that 6 of the 10 biomarkers in the study were equivalent between baseline and 25 and baseline and 50 FTC. These were CRP, IL-8, IL-10, IFN-γ, IP-10 and Eotaxin-1.
- MCP-1, MCP-2, TARC and GROα were not statistically equivalent between baseline and 25 and baseline and 50 FTC. However, effect sizes/ranks for MCP-1, MCP-2 and TARC were small, suggesting minimal difference between groups for these biomarkers.
- GROα was not stable across FTCs (see **Table 2**)

Table 3: Biomarker patterns across FTCs with participant age and sex as covariates

Biomarker	Participant Sex Baseline	Participant Sex 25 FTC	Participant Sex 50 FTC	Participant Age Baseline	Participant Age 25 FTC	Participant Age 50 FTC
CRP	β=1.29 p=0.02 [0.18,2.40]	β=1.12 p= 0.032 [0.094,2.14]	β=1.06 p=0.081 [-0.13,2.24]	β=-0.02 p=0.59 [-0.06,0.03]	β=-0.012 p=0.56 [-0.05,0.03]	β=-0.001 p=0.97 [-0.05,0.05]
IL-8	β=0.15 p=0.48 [-0.3,0.6]	β=0.48 p=0.02 [0.06,0.9]	β=0.16 p=0.5 [-0.3,0.6]	β=0.01 p=0.3 [-0.01,0.02]	β=0.01 p=0.19 [-0.01,0.03]	β=-0.003 p=0.7 [-0.02,0.012]
IL-10	β=-0.92 p=0.3 [-2.5,0.7]	β=-0.2 p=0.7 [-1.3,0.8]	β=-0.8 p=0.3 [-2.3,0.8]	β=-0.01 p= 0.6 [-0.02,0.03]	β=-0.01 p=0.7 [-0.03,0.02]	β=0.01 p=0.3 [-0.04,0.01]
IFN-γ	β=0.31 p= 0.03 [0.03,0.6]	β=0.24 p=0.13 [-0.07,0.6]	β=0.26 p= 0.08 [-0.03,0.6]	β=0.00 p=0.5 [-0.01,0.1]	β=0.00 p=0.6 [-0.01,0.1]	β=0.00 p=0.9 [-0.01,0.01]
IP-10	β=-0.4 p=0.5 [-1.6,0.7]	β=-0.2 p=0.6 [-1.1,0.7]	β=-0.18 p=0.7 [-1.1,0.8]	β= -0.02 p=0.2 [-0.05,0.011]	β= -0.01 p=0.4 [-0.04,0.02]	β= -0.01 p=0.4 [-0.04,0.02]
MCP-1	β=-45 p=0.16 [-108,17]	β=-32 p= 0.36 [-100,36]	β=-44 p=0.3 [-121, 34]	β=-0.1 p=0.92 [-1.97,1.77]	β=-0.004 p=1.0 [-2,2]	β=0.025 p=0.98 [-2.3,2.3]
MCP-2	β=-29 p=0.01 [-50,-8]	β=-30 p=0.00 [-49,-10]	β=-27 p=0.01 [-47,7]	β=-0.3 p=0.4 [-0.89,0.37]	β=-0.3 p= 0.3 [-0.88,0.28]	β=-0.3 p=0.3 [-0.89,0.31]
Eotaxin-1	β=0.25 p=0.5 [-0.5,1.0]	β=0.26 p= 0.5 [-0.4,0.9]	β=0.3 p=0.4 [-0.4,1.1]	β=-0.01 p=0.4 [-0.04,0.02]	β=-0.01 p=0.6 [-0.03,0.017]	β= 0.6 p= 0.6 [-0.04,0.02]
TARC	β=-0.29 p=0.7 [-1.70,1.11]	β=-0.23 p=0.78 [-1.83,1.37]	β=-0.08 p=0.9 [-2.07,1.92]	B=-0.037 p=0.94 [-0.08,0.00]	β=-0.04 p=0.07 [-0.09,0.00]	β=-0.06 p=0.2 [-0.12,-0.01]
GROα	β=-0.62 p=0.95 [-20.5,19.3]	β=-0.81 p= 0.96 [-34.5,32.9]	β= -1.79 p=0.94 [-37,33.7]	β=-0.15 p=0.6 [-0.78,0.48]	β=0.055 p=0.92 [-1.02,1.13]	β=-0.09 p= 0.87 [-1.15,0.97]

Table depicts β-coefficients, p-values and 95% CI

- Univariate generalized linear models for sex and age depict similarities and differences across FTCs for each covariate. For sex differences, it should be noted that CRP data consisted of 3 males out 17 total participants; all other data consisted of 2 males out of 12 total participants, 11 for IFN-γ(see Table 1)
- Patterns (direction of β-coeff., pval sig., 95% CI) for age are retained across FTCs for CRP, IL-8, IFN-γ, IP-10, MCP-2, Eotaxin-1 and TARC
- Patterns for sex are retained across FTCs for CRP, IL-10, IP-10, MCP-1, MCP-2, Eotaxin and TARC (see **Table 3**)

Discussion

- The study aimed to determine stability of serum biomarkers to determine the feasibility of research with specimens stored in laboratory freezers and bioarchives. While FTCs don't replicate long-term freezer storage, the study provides valuable information on potential biomarker degradation during specimen handling and storage.
- CRP, IL-8, IL-10, IFN-γ, IP-10 and Eotaxin-1 were robust from baseline to 50 FTC and may be ideal proteins for research.
- Although MCP-1, MCP-2 and TARC did not maintain equivalence across FTCs, a small effect size suggests differences are small, and may produce good results when specimens of similar age and storage method are compared.
- Of the 10 biomarkers, GROα, values showed strong evidence for lack of equivalence. However, GLM patterns were retained for this cytokine when participant sex was the covariate.
- Limitations of the study include small sample size and small number of male participants. A larger sample will help elucidate whether serum MCP-1, MCP-2 and TARC levels remain statistically equivalent across FTCs.