Determination of Interferons and Biomarkers in Influenza Donor Samples

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Introduction: Previous studies investigating biomarkers in Influenza have looked at a limited subset of markers with few studies examining Type I and II Interferon (IFN) concentrations in coordination with other downstream biomarkers or inflammatory cytokines. Until recently, measurement of Type I IFNs in the low or sub-pg/ml range was difficult, if not impossible. As a preliminary effort towards developing biomarker panels which can discriminate between viral and other infections and potentially discriminating between different viruses, we have examined a broad panel of biomarkers by immunoassays. These include IFNs Alpha (α), Beta (β), Omega (ω), and Gamma (γ). Downstream markers include CXCL-10, Neopterin, and Beta-2 Microglobulin (β2-M). Other Inflammatory markers included are IL-1 Beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-Alpha (α).

Materials and Methods: Influenza and Healthy Donor sera were obtained from BioIVT and BioChemed. Both sources have IRB approval for biomarker studies. Samples were aliquoted and frozen on arrival, and no more than 1 additional freeze thaw was performed before biomarker interrogation. IFN-α (PBL 41115), IFN-β (PBL 41415), IFN-γ (MSD K151QOD) Neopterin (IBL RE59321), Beta 2-Microglobulin (R&D KGE019) and were measured by singleplex ELISA. IFN-α, IFN-β, IFN-ω, IFN-γ, IFN-λ, IL-1α, IL-6, TNF-α, and CXCL-10 were measured by a plate based chemiluminescent multiplex assay (PBL 515000). IFN-γ, IL-1β, Il-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, CXCL-10, and TNF-α were analyzed through electrochemiluminescent multiplex assay (MSD K15049D and K15047D). All commercial assays were run by the manufacturers' protocols. IFN-ω immunoassay was developed using 2 monoclonal antibodies from Thermo-Fisher on the Simoa© platform using the homebrew assay development kit (Quanterix). The sensitivity of the final assay was 0.06 pg/ml.

Statistical Analysis: Donor classes (Healthy and Influenza) were defined based on the clinical diagnosis of disease state. Samples displaying a concentration below the analytical sensitivity of a given assay were assigned a corrected value based on the manufacturer's limit of detection claim. Biomarker populations were compared between stratified groups by Mann-Whitney U-Test. Correlations between different biomarker distributions for the Influenza cohort were compared under Spearman conditions and excluded samples which exhibited a double negative result on the respective assays. An alpha value of 0.05 was assigned during all statistical analysis to demarcate statistical significance.

Study Interpretations: Upregulation between Type I and II Interferons (IFN-α, β, γ, and ω) are prevalent and well-correlated during the incidence of Influenza. CXCL-10, TNF-α, IL-10, and Neopterin levels trended positively against the combined Interferon Score. A larger Influenza cohort can provide greater interpretations towards clinical biomarker crosstalk and disease prominence.

Selected References:
Llibre *et al*. Front. Cell Infect. Microbiol. 2019 Aug 22; 9:296. Alpha and Beta in Flu.
Pizzini *et al*. Influenza Other Respir. Viruses. 2019 Sep 6. Neopterin in Flu.
Hoffmann *et al*. Sci. Rep. 2016 Dec 6; 6:38532. IP-10, IFN-γ in Flu and Pneumonia.

Table 1. Demographics of Healthy and Influenza Cohorts.

	Healthy		Influenza	
Donors (n)	64		38	
Male (n)	25	39.1%	16	42.1%
Female (n)	39	60.9%	22	57.9%
Age	39.0±14.1		43.3±15.9	
Caucasian	40	62.5%	33	86.8%
Black	19	29.7%	4	10.5%
Hispanic	5	7.8%	0	0.0%
Other	0	0.0%	1	2.6%
Type A	0	0.0%	20	52.6%
Type B	0	0.0%	2	5.3%
Untested	64	100.0%	16	42.1%

Table 2. Biomarker, Statistical, and Readability Metrics among Cohorts.

	Healthy Median (g/l)	Influenza Median (g/l)	p value	Healthy % Read	Influenza % Read	Influenza Cut-Point (g/l)	Influenza Above CP
IFN-ω	6.00E-11	5.42E-10	<0.01	1.6%	68.4%	6.00E-11	68.4%
IFN-α	4.20E-10	1.51E-08	<0.01	0.0%	68.4%	4.20E-10	68.4%
IFN-β	1.00E-09	1.00E-09	<0.01	0.0%	39.5%	1.00E-09	39.5%
IFN-γ	1.97E-09	2.44E-08	<0.01	96.9%	100.0%	6.32E-09	78.9%
IFN Score	1.68E-09	2.72E-08	<0.01	---	---	2.13E-09	86.8%
CXCL-10	1.93E-07	1.42E-06	<0.01	100.0%	100.0%	7.13E-07	65.8%
IL-1β	1.00E-10	3.32E-10	<0.01	29.7%	73.7%	5.62E-10	31.6%
IL-2	1.91E-10	4.36E-10	<0.01	54.7%	92.1%	5.42E-10	28.9%
IL-4	4.00E-11	4.00E-11	0.25	3.1%	10.5%	4.00E-11	10.5%
IL-6	1.22E-09	3.51E-09	<0.01	100.0%	100.0%	3.36E-09	55.3%
IL-8	1.06E-08	3.01E-08	<0.01	100.0%	100.0%	2.22E-07	10.5%
IL-10	2.32E-10	1.03E-09	<0.01	93.7%	100.0%	6.72E-10	65.8%
IL-12p70	2.20E-10	2.20E-10	0.45	6.3%	10.5%	2.43E-10	7.9%
IL-13	4.80E-10	4.80E-10	0.92	45.3%	42.1%	1.79E-09	2.6%
TNFα	9.82E-10	3.23E-09	<0.01	100.0%	100.0%	3.02E-09	55.3%
Neopterin	2.26E-06	5.98E-06	<0.01	100.0%	100.0%	4.06E-06	65.8%
β2-M	1.86E-03	3.57E-03	<0.01	100.0%	100.0%	3.84E-03	47.4%

Figure 1. Scatter plots outlining the median and inter-quartile range of individual biomarker levels among healthy (gray, n=64) and influenza (red, n=38) cohorts.
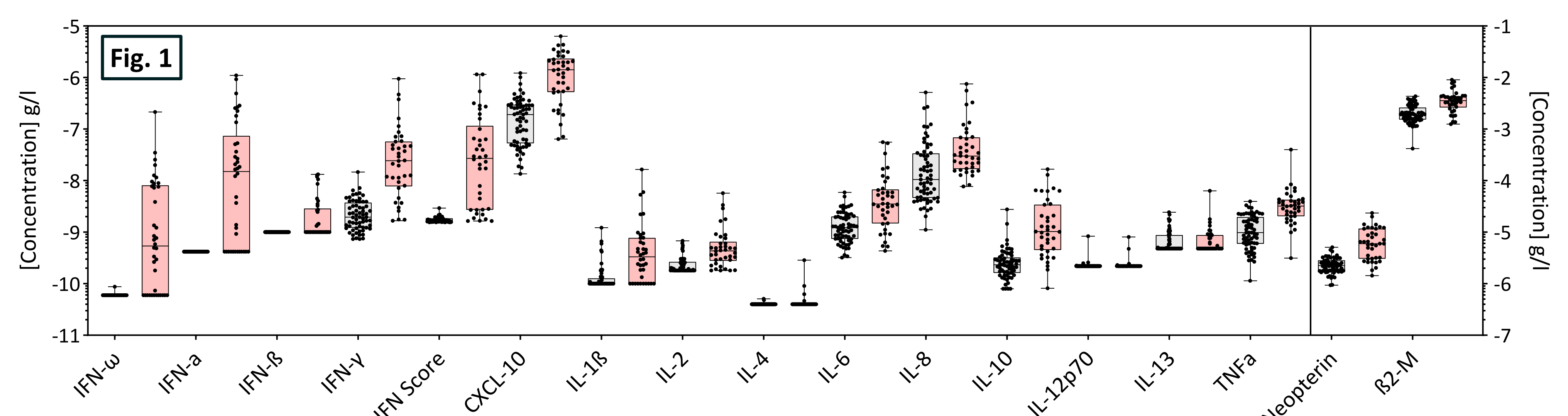
Figure 2. Heat map visualization of influenza ("flu") patient biomarker stratification at levels above (blue) or below (red) the healthy population cut-point (95th percentile).
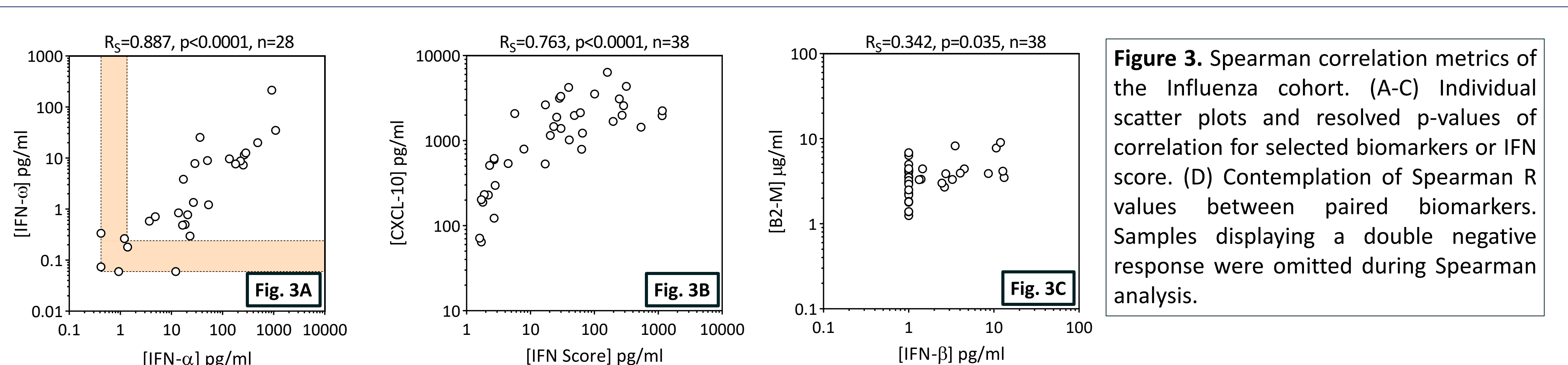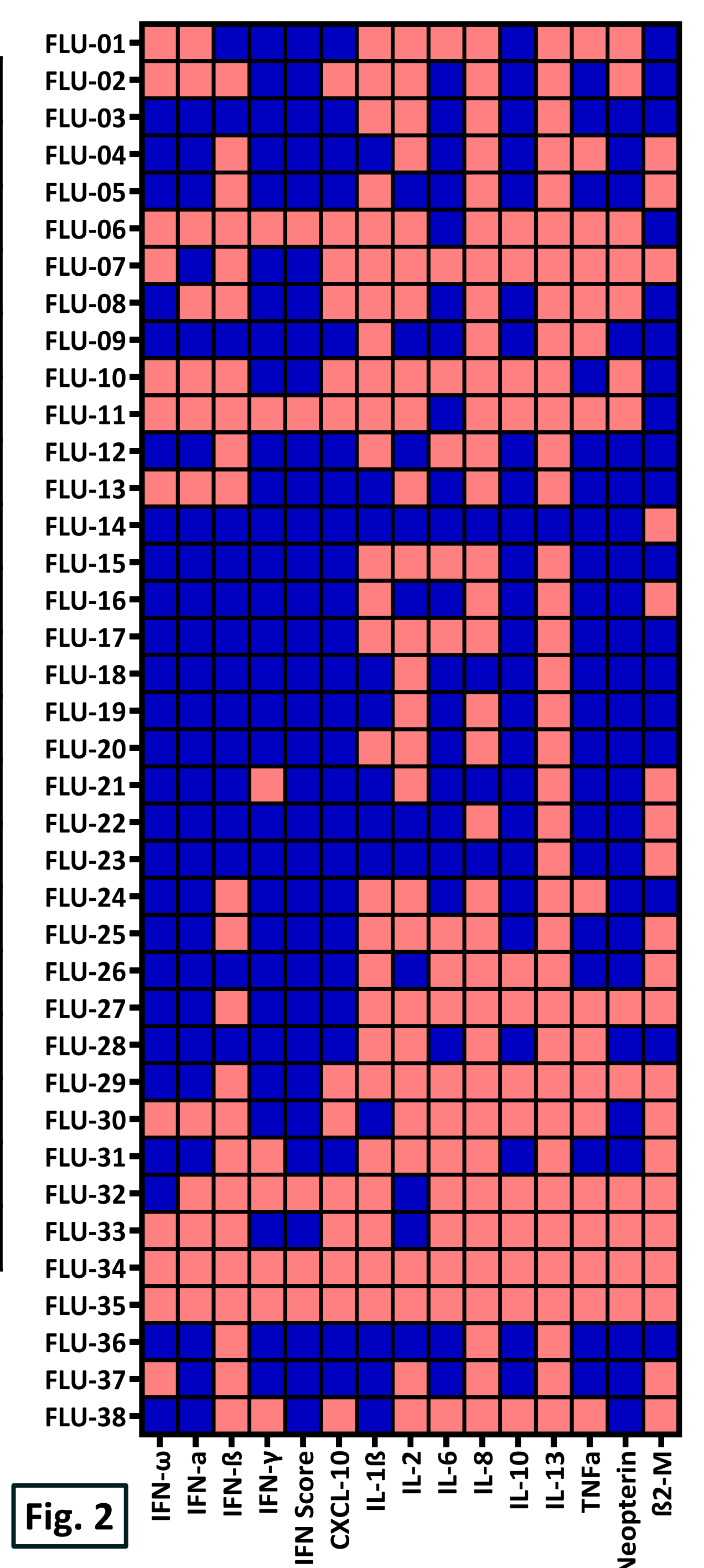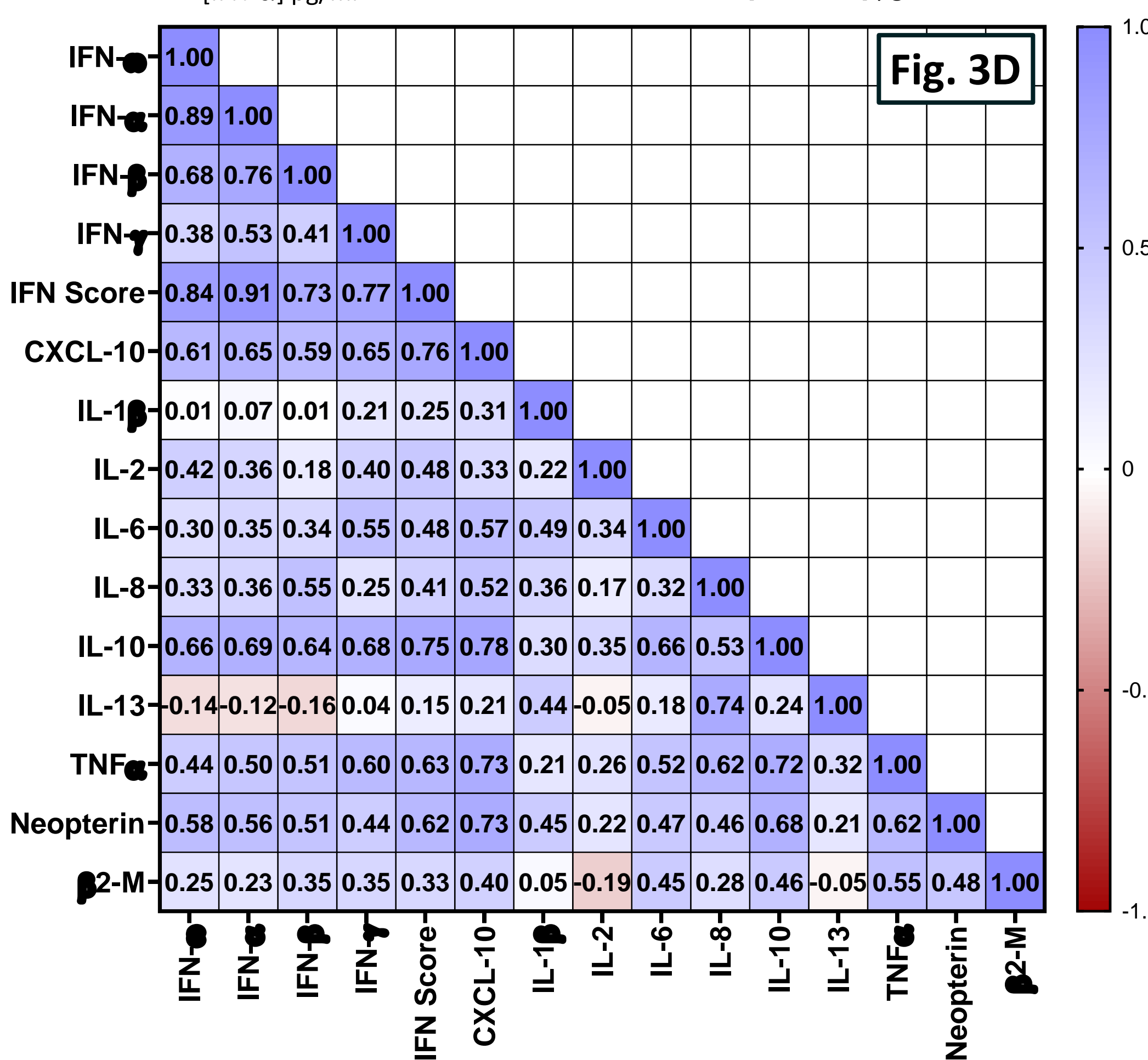

Figure 3. Spearman correlation metrics of the Influenza cohort. (A-C) Individual scatter plots and resolved p-values of correlation for selected biomarkers or IFN score. (D) Contemplation of Spearman R values between paired biomarkers. Samples displaying a double negative response were omitted during Spearman analysis.

Conclusions

- IFN-α and IFN-ω are highly correlated in Influenza patient sera.

- CXCL-10 correlates well with IFN-Score in Influenza.

- Little or no IFN-α, IFN-β, or IFN-ω is detected in healthy donors.

- Larger cohorts with more complete patient information should provide more robust results.

- Initial small studies with Hepatitis B and C donors show no Type I IFN.
  - Acute vs. chronic viral infection?