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A comparison of biofluid cytokine markers across platform technologies: Correspondence or divergence?

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Abstract

Background: Quantification of biofluid cytokines is a rapidly growing area of translational research. However, comparability across the expanding number of available assay platforms for detection of the same proteins remains to be determined. We aimed to directly compare a panel of commonly measured cytokines in plasma of typically aging adults across two high sensitivity quantification platforms, Meso Scale Discovery (MSD) and single-molecule immunosorbent assays (Simoa) by Quanterix.

Methods: 57 community-dwelling older adults completed a blood draw, neuropsychological assessment, and brain MRI as part of a healthy brain aging study. Plasma samples from the same draw dates were analyzed for IL-10, IP-10, IL-6, TNFα, and IL-1β on MSD and Simoa, separately. Reliable detectability (coefficient of variance (CV)<20% and outliers 3*upper interquartile range removed), intra-assay precision, absolute concentrations, inter-platform comparability, and concurrent associations with external variables of interest (e.g., demographics, peripheral markers of vascular health, and brain health) were examined.

Results: The proportion of cytokines reliably measured on MSD (87.7-93.0%) and Simoa (75.4-93.0%) did not differ (ps>0.32), with the exception of IL-1β which was only reliably measured using Simoa (68.4%). On average, CVs were acceptable at <8% across both platforms. Absolute measured concentrations were higher using Simoa for IL-10, IL-6, and TNFα (ps<0.05). MSD and Simoa shared only small-to-moderate amounts of variance with one another on the same cytokine proteins (range: r=0.26 for IL-10 to r=0.64 for IL-6), though platform agreement did not dependent on cytokine concentrations. Cytokine ratios within each platform demonstrated similar relative patterns of up- and down-regulation across MSD and Simoa. Supporting concurrent validity, all 95% confidence intervals of the correlations between cytokines and external variables overlapped between the two platforms. Moreover, most associations were in expected directions and consistently so across platforms (e.g., IL-6 and TNFα), though with a few notable exceptions for IP-10 and IL-10.

Conclusions: MSD and Simoa showed comparable detectability and intra-assay precision measuring a panel of commonly examined cytokine proteins, with the exception of IL-1β which was not reliably detected on MSD. However, Simoa demonstrated overall higher concentrations and the two platforms did not show agreement when directly compared against one another. Relative cytokine ratios and associations demonstrated similar

patterns across platforms. Absolute cytokine concentrations may not be directly comparable across platforms, may be analyte dependent, and interpretation may be best limited to discussion of relative associations.

Introduction

Proteomic markers are a rapidly growing area of clinical research to help characterize the biologic cascades underlying disease and behaviors, and improve monitoring and diagnostics in humans. Biofluid cytokine concentrations are a particularly widely measured, diverse group of proteins used to estimate immune activation processes in various healthy and diseased populations; a tool that now spans across disciplines (e.g., psychiatry, neurology) and in large multisite clinical studies[1]. Advancements in technologies have facilitated ease and pragmatics of biofluid marker quantification by allowing for multiple simultaneous marker analysis via multiplexing, higher analytical sensitivity and lower limits of detection and quantification, and reduction in the amount of sample needed (e.g., compared to standard ELISA)[2–4]. However, the growing number of available platforms with slightly differing technologies measuring the same protein markers has made it difficult to not only select the most optimal quantification tool, but to compare marker results across platforms and studies. Though tempting to interpret these "biologic data" as free from external biases, the reproducibility and reliability of the same protein across each platform may not be one-to-one[5]. These issues become especially relevant in the context of multisite (i.e., multiple laboratories) and longitudinal studies, and for cytokine measurement, which are posited to reflect more nuanced and potentially rapidly changing immune cascades with differing levels of detectability, potentially heightening any (even minute) differences in platform technologies. As the field continues forward with these exciting tools to probe into molecular mechanisms in humans, a more comprehensive and empirical characterization of distinct proteomic marker approaches will be critically important.

While a host of studies have examined platform differences in cytokine measurement using pooled control and known cytokine concentration spiked samples under highly controlled protocols, much fewer have conducted these direct comparisons in actual patient populations. Even using the former standardized spiked samples approach, distinct differences across platforms emerge. Meso Scale Discovery (MSD) and Biosource Luminex appear to demonstrate overall comparable spike recoveries, coefficients of variance, and low limits of detection, though even these findings may be analyte dependent[6–10]. For instance, in a direct comparison study by Fu and colleagues, in unspiked serum, MSD showed better performances for IL-10, TNFa, and IL-2, IFNy, and CSF-GM, Luminex showed better performances for IL-8, and the platforms appeared comparable for IL-6[8]. Indeed, the authors concluded that no single assay platform can likely be used for all cytokine

quantifications, and that optimal assays and platforms must be selected by cytokine of interest. The comparability of cytokine measurements across platforms may be even less clear when examining patient biofluids who have unknown levels of immune activation and countless potentially contributing factors on cytokine expression. Despite the inherently less controlled nature of patient platform comparison studies, understanding how distinct proteomic platforms perform in such “real world” samples is needed to support the ecologic validity and translation of biofluid marker technologies for human application. In one study, Breen et al. examined 13 cytokine analytes on two platforms (MSD and Luminex) across six laboratory sites as part of the Multicenter AIDS Cohort Study and Women Interagency HIV Study[1]. Every cytokine examined demonstrated at least one significant lab and/or assay lot effect. Only IL-6 was directly compared on the same samples across platforms and demonstrated correlations with standard ELISA varying between r=0.13 to 0.79 in serum and plasma with MSD or Luminex (from Bio-Rad, BioSources, and Linco manufacturers) platforms. Notably, however, there was convergence of the relative patterns of cytokine elevations across platforms, lots, and laboratories in HIV infection, including measurable cytokine increases in pre-/post-HIV viremia and significant differences between seropositive and negative individuals. Interestingly, other studies that have examined serum cytokine concentrations in clinical HIV[6] and aging and Alzheimer’s disease[5] demonstrate moderate to high correlations across platforms (HIV: IL-6 and IL-8 r’s=0.62 to 0.80; aging/AD r’s=0.60 to 0.97), and fairly consistent diagnostic group differences. Given that anywhere from <2% to 94% of the variance in cytokine concentration may be explained by platform approach, this appears to be highly dependent on the analyte measured, and the growing number of new platforms, continued data quantifying when these differences may be expected are needed to make sense of biofluid proteomic data moving forward.

One such newer biofluid protein analysis technology is single-molecule enzyme-linked immunosorbent assays (Simoa) by Quanterix. Simoa are bead-based ELISAs consisting of high-density microarrays with femtoliter-sized wells each containing an individual bead (single- or multi-plexed). Simoa was designed using this microscopic bead technology with the goal of increasing sensitivity of detection to low concentration proteins. During analysis, beads containing an enzyme-labeled immunocomplex are hydrolyzed into a fluorescent product that can then be quantified as the proportion of wells that generate a light signal[3]. Though both based on ELISA techniques, this technology differs slightly from the more extensively utilized Meso Scale Discovery (MSD) platform, which utilizes an electrochemiluminescence detection system. In MSD, plate wells

are fitted with carbon electrodes that are coated with different capture-antibodies of interest. Following standard sandwich ELISA techniques and using a ruthenium-conjugated secondary antibody, upon electrical stimulation, the ruthenium chemical label emits light at the surface of each analyte-specific electrode. Light intensity is then measured per electrode to estimate analyte concentrations. While MSD has been more comprehensively studied and demonstrates comparable or better sensitivity and intra-assay precision compared to other available platforms, due to its single-bead femtomolar-sized wells technology, Simoa requires less sample and purports to be more sensitive to proteins at very low concentrations. While a handful of studies have demonstrated superiority of Simoa compared to standard ELISA in these respects, very few have compared MSD to Simoa. In one study, Myzithras and colleagues found Simoa achieved higher sensitivity detecting a growth factor (GDF11), a 100-fold improvement in the lower limit of quantification compared to MSD, in standardized pooled samples[11]. Another study demonstrated that use of CSF AB42/Ab40 ratios as quantified on MSD or Simoa were comparably sensitive in classifying abnormal amyloid PET[12]. However, no clinical studies have systematically compared MSD and Simoa across a panel of inflammatory cytokines.

Given the scientific and clinical potential of proteomic markers, technological availability including MSD (widely validated) and Simoa (high sensitivity to otherwise unmeasurable molecules), and yet apparent analyte- and platform-specific quantification effects, pragmatic guidelines regarding platform selection per analyte are needed. We aimed to characterize the comparability of commonly measured inflammatory cytokine markers drawn from typically aging adults analyzed across MSD versus Simoa. We directly examined intra-assay precision, reliable detectability, and examine concurrent validity via relative profiles of associations with external measures of vascular and brain health across MSD and Simoa techniques.

Methods

Participants. 57 typically aging, community-dwelling older adults completed a blood draw that was analyzed on both MSD and Simoa platforms, as well as neuropsychological and neurological evaluations and a brain MRI as part of a larger healthy aging study at the University of California, San Francisco Memory and Aging Center (see Table 1). Inclusion criteria at study visit were a neurologic and neuropsychological exam within normative standards per consensus research criteria[13], no major memory concerns or diagnosed memory

condition, and no major medical/psychiatry condition that may affect cognitive functioning (e.g., schizophrenia, epilepsy).

Table 1. Demographic and clinical characteristics of typical aging adult study sample (N=57).

Age, y	78.2 (6.4), range 64-95
Education, y	17.9 (2.0), range 12-20
Sex (%F)	57.9% (33)
Race % White % Asian % Other/Unknown	 93.0% (53) 3.5% (2) 3.5% (2)
MMSE (median, IQR)	29 (28, 30), range 25-30
Modified Trail Making Test (# sequences/second)	0.64 (0.22)
Corpus Callosum FA	1.6 (0.13)
Body Mass Index	26.5 (4.2), range 19.9-37.2
Triglycerides (mg/L) (n=35)	84.5 (39.7), range 31-186
HOMA-IR (n=38; median, IQR)	1.9 (1.3, 2.8), range 0.7-8.7
C-reactive protein (n=38; median, IQR)	1.05 (0.38, 1.8), range 0.1-18.6

Note. MMSE = Mini-Mental State Examination; IQR = inter-quartile range; FA = fractional anisotropy; HOMA-IR = homeostatic model assessment of insulin resistance.

Cytokine Measurement. After a 12-hour overnight fast, blood was drawn in the morning and centrifuged at 2000x g for 15 minutes at 4°C before being transferred into 500µL polypropylene cryovials for storage. Samples had never been thawed prior to analysis and were gradually brought to room temperature for analysis (described below). Plasma collected from the same participants on the same day were then analyzed for interleukin 10 (IL-10), interferon gamma-induced protein 10 (IP-10; CXCL10), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), and interleukin 1 beta (IL-1β) on Meso Scale Discovery and Simoa platforms, separately. All analyses were conducted by a board-certified laboratory technician blinded to study design.

Meso Scale Discovery (MSD). Plasma cytokine concentrations were obtained using the Human Proinflammatory Panel 1 V-PLEX (IL-10, IL-6, TNFα, and IL-1 β) and Human Chemokine Panel 1 V-PLEX (IP-10). Assay analyses were conducted in accordance with the manufacturer's protocol. Each sample was measured in duplicate with manufacturer provided standards per plate. The multiplex arrays are scanned via the Mesoscale QuickPlex SQ 120 and cytokine concentrations were quantified using the manufacturer provided software, Discovery Workbench v4.0, based on sample dilution and relative to the supplied in-assay standard curve.

Simoa by Quanterix. The Cytokine 3-Plex A (IL-10, IL-6, TNFα) and single-plexes of IP-10 and IL1b were used to obtain cytokine concentrations using Simoa. Analyses were performed according to

manufacturer's protocol, measured in duplicate per plate, and scanned and quantified by the Simoa HD1-Analyzer.

Demographic and Clinical Variables of Interest. Data were additionally gathered on several external variables of interest to serve as object indicators of relative construct validity and comparability cytokine marker associations across platforms. Given this analysis was conducted within the context of a healthy brain aging study, we selected to examine the relationship between cytokine markers across MSD and Simoa with age, sex, education, cognition, white matter integrity, and several peripheral measures of vascular health.

Cognition. Participants completed the Mini-Mental State Examination as a global indicator of cognition[14]. Additionally, speeded executive control was measured utilizing a modified version of the Train Making Test which required participants to serially alternate between numbers and days of the week. The total number of sequences completed was divided by the time to complete, such that higher values indicate better cognitive control. We elected a measure that taps into both processing speed and executive functions given that these abilities are most strongly and consistently associated with inflammatory markers among both healthy older adults and in neurodegenerative syndromes[15].

White Matter Integrity. Given the established animal studies and growing clinical literature implicating the role of immune activation in white matter health (via both potentially vascular pathways and primary neurodegenerative pathologies)[16,17], we aimed to additionally characterize the relative profile of associations between our cytokine markers and FA of the corpus callosum across MSD and Simoa.

MRI acquisition. Magnetic resonance imaging (MRI) scans were obtained using a Siemens 12-channel head coil and were performed on a 3 Tesla Siemens Tim TrioMR scanner at the UCSF Memory and Aging Neuroscience Imaging Center. Whole brain T1 images were acquired using Magnetization-prepared rapid gradient echo (MPRAGE) in the axial plane: TR=2300ms; TE=3.43ms; TI=900 ms; flip angle=9; slice thickness=1 mm; FOV=256*224 mm; voxel size=1 mm*1mm; matrix size=256*224; and number of slices=176. Diffusion Weighted Images (DTI) were acquired using single-short spin-echo sequence with the following parameters: TR=5300 ms; TE=88 ms; TI=2500 ms; flip angle=90; FOV=256*256 mm; two diffusion values of b=0 and 1000 s/mm; 12 diffusion directions; four repeats; 40 slices; matrix size=128*128; voxel size=2 mm*2 mm; slice thickness=3 mm; and GRAPPA=2.

Image processing and analysis. We analyzed fractional anisotropy (FA) as the primary parameter of interest from diffusion weighted images as a marker of white matter tract microstructure. FSL software co-registered the diffusion direction images with the b=0 image, then applied a gradient direction eddy current and distortion correction. Diffusion tensors were calculated using a non-linear least-squares algorithm from Dipy. After quality control, patients' tensor (four-dimensional image) were registered linearly and non-linearly into a common space using DTI-TK. At baseline, patients' tensors were moved into a group template. DTI scalar maps (fractional anisotropy, mean diffusivity) were calculated from the patients' tensor in the group template space. White matter tracts were masked using the ICBM-DTI-81 white matter labels and tract atlas[18]. We then selected the corpus callosum as our region of interest to reflect a global metric of white matter integrity, given that it comprises tracts from many cortical regions and appears to be a robust marker of diffuse white matter changes.

Peripheral Vascular Health. Blood collected from the fasting morning draw was also analyzed for clinical labs in a subset of individuals (n=38), including serum levels of C-reactive protein, insulin, glucose, and triglycerides using standard laboratory protocols. C-reactive protein (CRP) was selected as a comparison of a widely used clinical indicator of “pro-inflammatory state.” A homeostatic model assessment of insulin resistance (HOMA-IR) was calculated in mass units [(glucose*insulin)/405] to provide a global estimate of insulin resistance at the time of blood draw. Body mass index was also calculated at the time of blood draw. These clinical markers of vascular health were selected given their consistently reported association with pro-inflammatory cytokine elevations in serum and plasma[19,20], and to provide more direct comparison between peripheral cytokines and peripheral indicators of clinical health (versus cognition or brain structure, which reflect central processes).

Statistical Analyses

To identify cytokine values that were reliably measured, we first removed values with CVs>20%. We then removed outliers defined as 3 times the sample upper quartile range value for each marker. We compared these final proportions of “reliably detectable” values across MSD and Simoa via McNemar’s test of symmetry for paired nominal data.

For subsequent analyses, all cytokines and CV values were log transformed to allow for parametric analyses. Matched-pairs t-tests were then conducted to examine the comparability of CVs and absolute values

(pg/mL) across platforms. Pearson correlations were calculated to estimate the agreement, and Bland-Altman plots were developed to examine differences between MSD and Simoa measurement techniques across each cytokine marker. Additionally, to inform future studies using pathway analyses examining relative cytokine concentrations across a panel of proteins, we explored relative ratios of cytokine concentrations within each platform; given that some markers had negative log values, we added an integer (log+3) to all values to depict positive ratios. Finally, Pearson correlations were also used to examine the relative effect sizes of each cytokine marker with external variables of interest across MSD and Simoa.

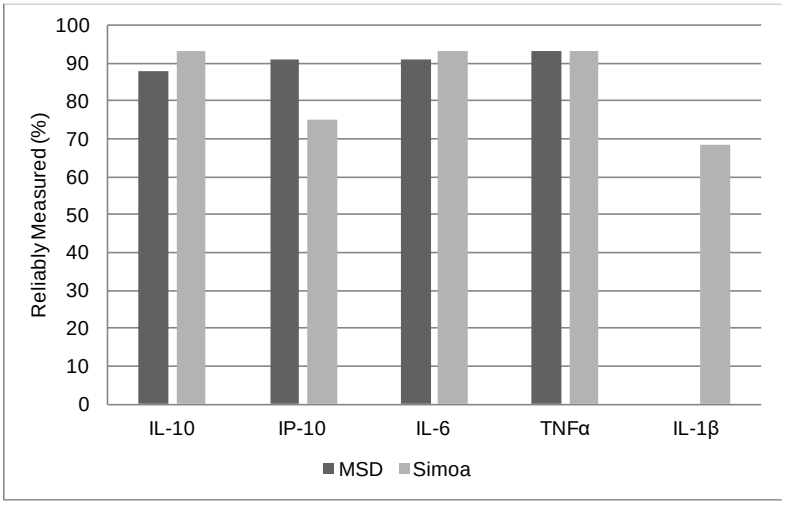
Results

Reliable Detectability, Intra-assay Precision, and Absolute Concentrations.

IL-1β. While IL-1β was reliable detected for 68.4% of the sample on the Simoa platform, none of the IL-1β MSD values met our reliable detectability operationalization (CVs range 35.0-131.4%). Therefore, our subsequent platform comparison analyses were restricted to IL-10, IP-10, IL-6, and TNFα.

IL-10, IP-10, IL-6, TNFα. Applying the reliability parameters outline above (CV<20% and outliers 3*upper IQR removed), of the 57 older adult samples on which we attempted to measure cytokine concentrations, MSD resulted in 87.7-93.0% reliable detectability while Simoa resulted in 75.4-93.0% reliable detectability (Figure 1). The MSD and Simoa platforms did not significantly differ in terms of reliable data produced (McNemar's test ps>0.32).

Figure 1. Proportion of samples reliably measured (i.e., CVs<20 and outliers 3*upper IQR removed) across cytokine markers on Meso Scale Discovery (MSD) and single molecule array (Simoa) platforms did not differ, with the exception of IL1β.



On average, both platforms demonstrated CVs within the acceptable range <8%. However, Simoa demonstrated a significantly higher CV IP-10 (t(43)=3.4, p=0.001), while MSD demonstrated a significantly higher CV for IL-10 (t(48)=-2.6, p=0.01). CVs were statistically comparable for IL-6 (t(49)=0.72, p=0.48) and TNFα (t(49)=1.6, p=0.11) (Figure 2). To increase comparability of interpretation, all subsequent analyses were conducted among participants with reliably detectable data on *both* Simoa and MSD (IL-10 n=47; IP-10 n=39; IL-6 n=48; TNFα=49). The Simoa platform resulted in higher absolute levels (pg/mL) of IL-10 (t(46)=40.9, p<0.001), IL-6 (t(47)=11.2, p<0.001)), and TNFα (t(48)=2.8, p=0.007) compared to the MSD platform (Figure 3). Simoa and MSD did not significantly differ in absolute measurement of IP-10 (t(38)=1.9, p=0.07).

Figure 2. Average coefficients of variance (CV) across Meso Scale Discovery (MSD) and single molecule array (Simoa) quantification approaches are within acceptable ranges.

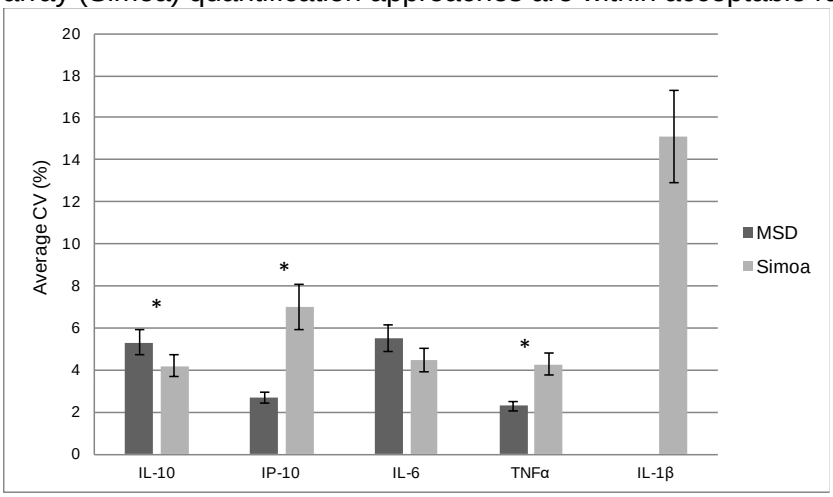
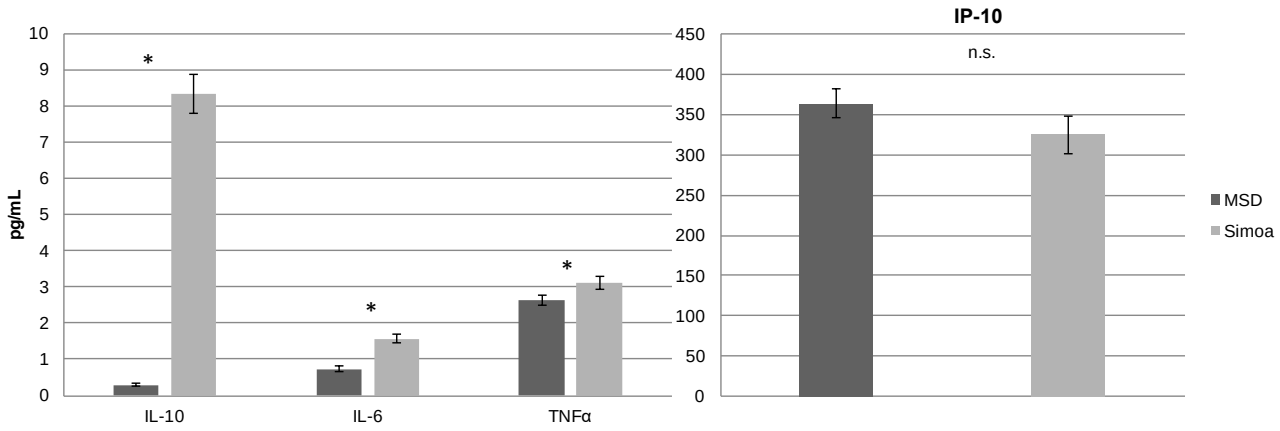


Figure 3. Absolute concentrations (pg/mL) differ between Meso Scale Discover (MSD) and single molecule array (Simoa) platform measurements for IL-10, IL-6, and TNFα.



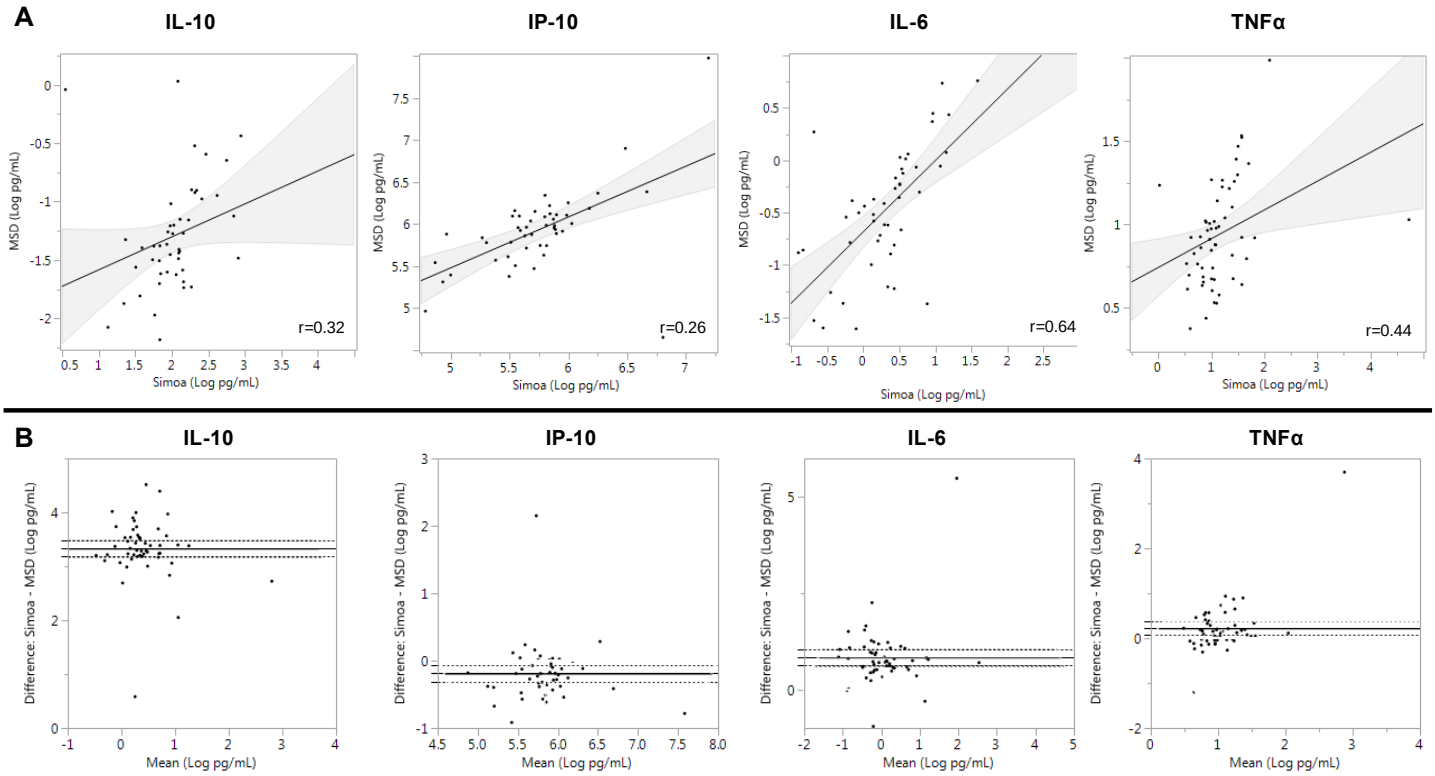
Inter-platform Comparisons.

Cytokine concentrations of the same protein marker demonstrated only small-to-medium associations across platforms. Though all statistically significant (ps<0.05), IL-6 demonstrated the strongest (r=0.64) and IL-10 the weakest correlation (r=0.26) between MSD and Simoa platform measurements (Figure 4a). Given concerns that restricted range in a healthy cohort may artificially contribute to weaker correlation values, we also examined correlation coefficients among participants with outliers included (IL-10 n=49, IP-10 n=40, IL-6 n=50, TNFα n=50), but CVs>20% removed; however, the range of Pearson correlation coefficients remained comparable (r range = 0.29 to 0.56).

To more directly examine platform differences and bias, Bland-Altman plots were developed. Simoa produced overall higher quantifications than MSD as indicated by the positive solid mean difference lines (Figure 4b), with the exception of IP-10, regardless of overall concentration levels (e.g., Simoa was not biased by those with overall higher values). Less than 95% of the sample fell within the 95% confidence intervals (dotted lines) for all markers examined suggesting significant differences between platforms; however, there did not appear to be a systematic bias in directionality. That is, most data points fell within and closely outside the 95% confidence intervals with about the same amount falling above and below the mean difference line.

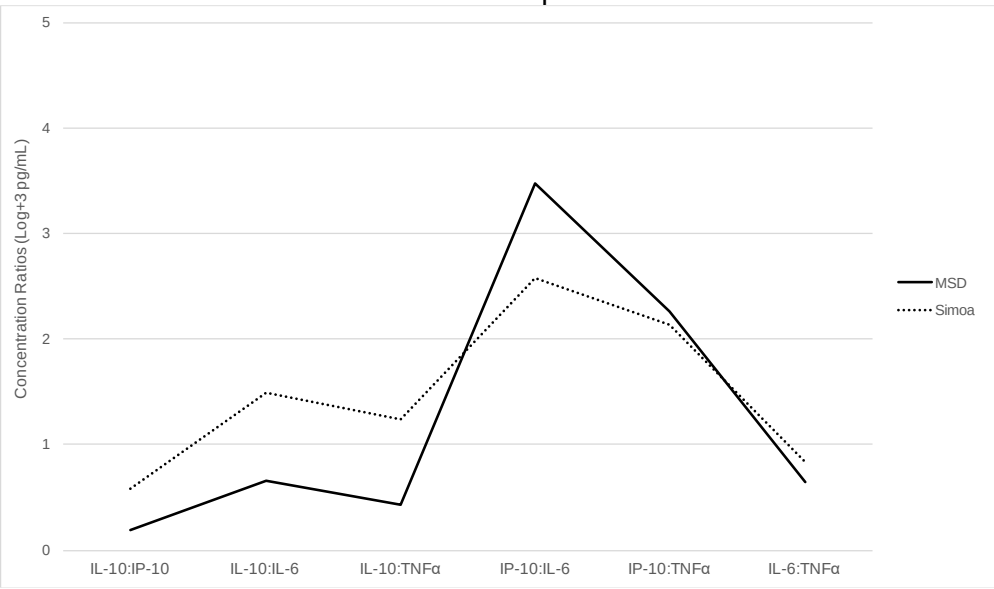
Additionally, given that panels of proteomic markers are commonly examined *relative to one another* to indicate aberrant up- or down-regulation within a postulated biological pathway, we explored the pattern of relative cytokine concentration ratios within each platform. Figure 5 demonstrates that, relative to the other measured cytokines, MSD and Simoa demonstrate a similar pattern of within-platform cytokine increases and decreases across the panel examined.

Figure 4. Cytokine concentrations are significantly, but not strongly correlated with each other and demonstrate significant differences as quantified by Meso Scale Discovery (MSD) versus Quanterix single molecule array (Simoa) platforms.



Note. A) Scatterplots depicting agreement between Simoa (x-axis) and MSD (y-axis); **B)** Bland-Altman plots illustrating platform differences. The y-axis represents the mean differences between MSD and Simoa, while the x-axis depicts the average between the platforms. The solid horizontal lines indicate the average difference and the dashed horizontal lines represent the 95% confidence intervals.

Figure 5. Ratios of cytokine concentrations demonstrate a similar pattern of relative increases and decreases between markers measured on the same platform.

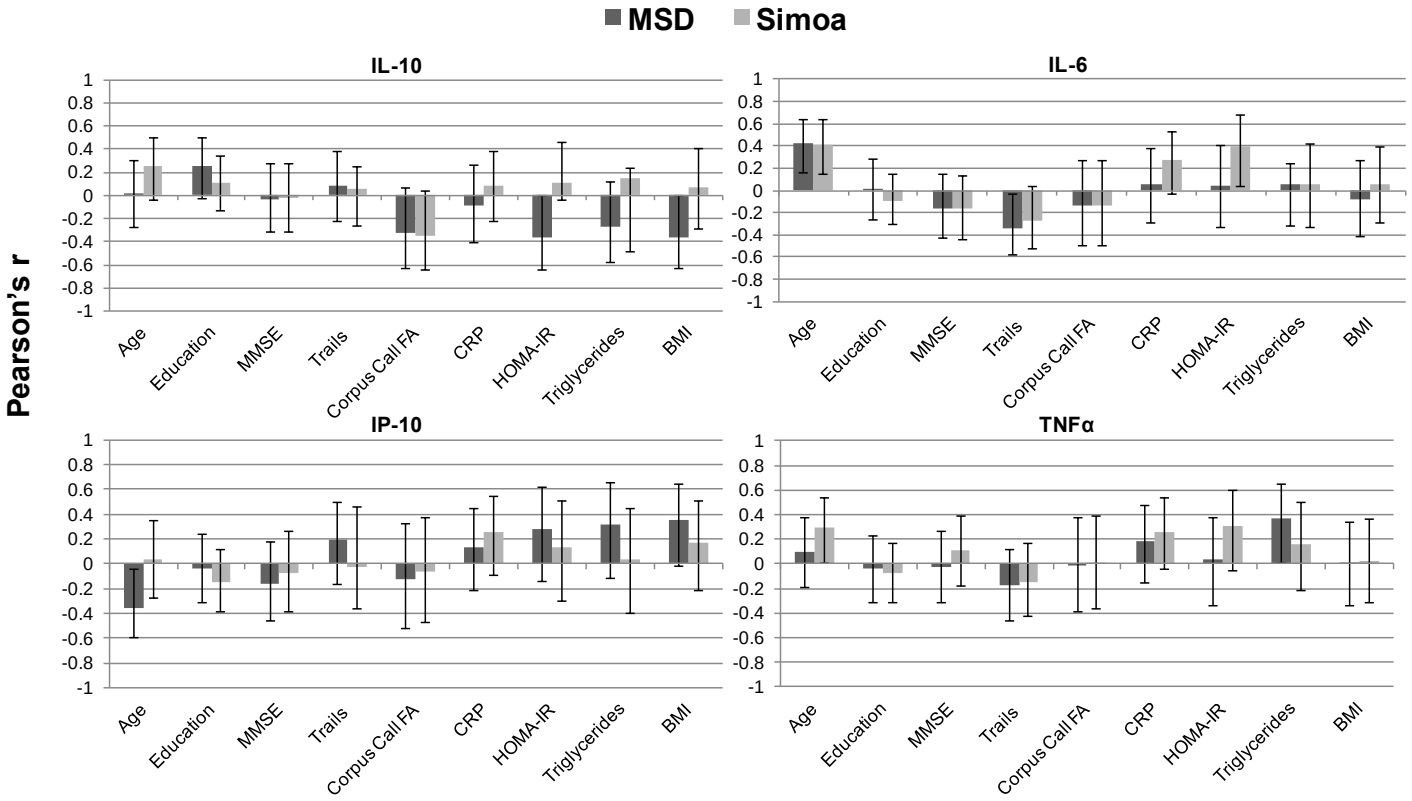


Note. Y-axis represents cytokine concentration ratios (log transformed + 3 in pg/mL). MSD = Meso Scale Discovery; Simoa = Quanterix single molecule array.

Inter-platform Correlations with Clinical and Demographic Variables.

Lastly, we examined the relative effect sizes of each cytokine marker with variables of interest (e.g., age, cognition, peripheral measures of vascular health) across the two platforms. As a whole, 95% confidence interval bars of the associations between the cytokines and external variables overlapped between the two platforms for all variables examined (Figure 6). While the majority of associations between external variables and the cytokine markers demonstrated the same (and expected) directionality across MSD and Simoa, there were several notable exceptions. IL-10 demonstrated the most variability of this nature across platforms. For example, though IL-10 is generally considered to promote anti-inflammatory cascades, it demonstrated a positive association with age across both MSD (r=0.01) and Simoa (r=0.25); additionally, though the error bars overlapped and associations were small, IL-10 measured via MSD showed negative associations with HOMA-IR (r=-0.36), triglycerides (r=-0.27), and BMI (r=-0.36), but positive associations with these variables as measured via Simoa (r's range=0.07-0.15). Notably, IL-6 again appeared to demonstrate the most consistent associations with variables of interest across the two platforms, both in terms of magnitude and direction of the effect, followed by TNFα. IP-10 generally demonstrated larger effect sizes with external variables when measured via MSD compared to Simoa; however, some associations were in unexpected directions. For example, IP-10 is implicated in the acute pro-inflammatory phase but demonstrated a significant negative association with age (r=-0.35) and a positive association with an executive functioning task (Trails r=0.20) when measured on the MSD platform. None of the cytokine markers across either platform were significantly associated with sex (t-ratio range = 0.4 to 1.6, ps>0.12) or education (r's range = -0.08 to 0.25, ps>0.08) in our sample.

Figure 6. Correlations (r and 95% error bars) between each measured cytokine and external variables of interest across Meso Scale Discovery (MSD) and single molecule array (Simoa) platforms.



Note. MMSE = Mini Mental Status Exam; Corpus Call FA = corpus callosum fractional anisotropy; CRP = C-reactive protein; HOMA-IR = homeostatic model assessment of insulin resistance; BMI = body mass index.

Discussion

Ours are the first data directly comparing cytokine measurement in clinical samples between Meso Scale Discovery (MSD) and single molecule arrays (Simoa) by Quanterix with the aim of helping to guide platform selection and future cross-study interpretations for several commonly examined immune activation markers. Although both predicated on ELISA techniques, distinct differences in cytokine quantification emerged. While the overall ability of each platform to detect our cytokines of interest was largely comparable (with the exception of IL1b), absolute concentrations significantly differed, and only moderate to minimal variance was shared across the platforms when measuring the same markers in the same blood samples. However, patterns of relative cytokine ratios and external associations *within platform* demonstrated greater similarities, suggesting that relative (versus absolute) cytokine concentrations relationships may be most appropriate when examining across measurement platforms. As interest in biofluid proteins continues to expand, despite representing postulated biological processes, it is important to appreciate that measurement of

cytokine activation may not be entirely “objective” and is in fact subject to common pitfalls of differences in any indirect assessment approach.

Given that the two measurement approaches do differ slightly, it may not be surprising that distinct differences in cytokine quantification emerged. That is, while MSD is an electrochemiluminescence technique utilizing electrodes coated in capture-antibodies that measures fluoresce signal produced by an electric-chemical reaction, Simoa consists of a bead-based enzyme-labeled immunocomplex that is chemically broken down into a measured fluorescent product. MSD quantifies the intensity of the fluorescence signal and Simoa counts the proportion of wells with a fluorescence signal.

With the exception of IL-1β, both MSD and Simoa demonstrated acceptable rates of quantification and intra-assay precision. Overall, the panel of cytokines was reliably measured (i.e., CVs<20% and outliers excluded) for ~90% of samples attempted with overall CVs <8% on both platform; though not significant, however, it is notable that IP-10 was only reliably measured in 75% of the sample using Simoa (versus 91% on MSD). The major exception to these comparable results was IL-1β, which only resulted in values for 3 out of the 57 attempted samples using MSD, none of which met our operationalization of reliability (CVs>34%). On the other hand, Simoa reliably measured IL-1β in 68% of samples attempted. Given that IL-1β may be present only at very low concentrations, particularly among healthy individuals, detectability is a clear challenge. Our results demonstrating the relatively poor sensitivity of MSD for IL-1β are consistent with previous studies examining this cytokine in patient samples, including HIV infection[1,6], multiple sclerosis[10], and kit control samples[8]. IL-1β is a key mediator in the inflammatory response and is involved in a host of cell functions (proliferation, differentiation, apoptosis), including exacerbates damage during states chronic disease and injury[21]. Given its ubiquity in immune activation, it is promising that Simoa was able to quantify IL-1β in >60% of the samples and perhaps represents advancement of biofluid technologies moving forward. However, validation that these concentrations are reliably measured across time and indeed represent the molecule of interest is still needed.

The major differences in MSD versus Simoa emerged when directly comparing cytokine concentration measurements head-to-head. Overall, Simoa quantified significantly higher concentrations particularly of IL-10 (27-fold higher), but also for IL-6 and TNFα (1-2 fold higher). Though it is unclear which platform represents “true” biologic levels, these findings are consistent with Simoa’s purported goal of increasing overall

measurement sensitivity (i.e., detection of “subfemtomolar concentrations” via femtomolar-sized wells). These differences in absolute cytokine values are consistent with almost all previously reported platform comparisons studies found, such that regardless of platforms examined, the resulting pg/mL concentrations do not appear directly comparable[1,10,22]. Additionally, directly correlating on the same cytokine markers, though all statistically significant, MSD and Simoa demonstrated only small-to-moderate effect sizes. Notably, IP-10 and IL-10 demonstrated the weakest ($r_s<0.35$), while IL-6 and TNFα demonstrated the strongest ($r_s>0.40$) shared variance. Similarly, Bland-Altman plots indicated that the two platforms significantly differed, with Simoa demonstrating a positive bias for IL-10, IL6- and TNFα, though the biases appeared to be regardless of the overall concentration levels (i.e., Simoa did not demonstrate disproportionately higher quantifications for individuals with the highest concentrations).

The reason(s) for these quantification differences is difficult to determine, though it is important to keep in mind that MSD and Simoa do differ in their quantification approaches. That is, while MSD is an electrochemiluminescence technique utilizing electrodes coated in capture-antibodies that measures fluoresce signal produced by an electric-chemical reaction, Simoa consists of a bead-based enzyme-labeled immunocomplex that is chemically broken down into a measured fluorescent product. MSD quantifies the intensity of the fluorescence signal and Simoa counts the proportion of wells with a fluorescence signal. Additionally, differences in the company-supplied antibodies, dilution protocols, and overall sensitivity and reliability of instruments may have contributed to our findings. Another likely reason for the different absolute concentrations across the platforms is that the assays have not been standardized to each other using common calibrators. It is also possible that the biology underpinning these cytokines play a role in their detection reliability, as well. That is, IL-6 and TNFα can be considered as wide-reaching master regulators of immune activation involved in both the initiation and initiated by most adaptive and innate inflammatory pathways[23–25]. As such, perhaps it is not surprising that they are among the most commonly reported cytokines indicating the most robust, reliably measured results across patient samples, in parallel to our findings here. These signaling proteins may therefore be particularly helpful to indicate an overall “pro-inflammatory state” but are less nuanced in their ability to implicate specific immune pathways. On the other hand, IP-10 and IL-10 represent relatively more specific immune activation pathways in the patient population or construct of interest, including acutely heightened immune responses and anti-angiogenic processes (IP-10) and anti-inflammatory

clearance (IL-10), including inhibiting activity of Th1 cells, NK cells, and macrophages[26–29]. Perhaps due to the greater subtleties of these pathways, IP-10 and IL-10 were more sensitive to even the small measurement approach differences examined here and may therefore be less reliable overall when compared across differing platform techniques. Notably, ours is among the few studies that have reported such direct platform comparisons in a patient sample (i.e., same plasma on two different platforms) and the only that has done so on MSD versus Simoa. Of those that have, correlations have ranged from 0.1 to 0.8[5,6], paralleling our findings and indicating that direct platform comparability of biofluid markers may be highly analyte specific.

However, cytokine ratios and associations with external variables within each platform demonstrated similar patterns, supporting *relative* interpretations of cytokine concentrations across platforms. Regarding the cytokine ratios, our data support the development of proteomic *profiles* within a targeted pathway as useful application of biofluid markers to help characterize a molecular process of interest, and may be less susceptible to the interpretation limitations inherent when examining a single marker, particularly across platforms and/or studies. That is, characterization of relative patterns of marker up- or down-regulation may be the most prudent interpretation of biofluid markers, rather than attempting to interpret the value of a single marker in the absence of the relative proteomic milieu. Additionally, by exploring the variance explained by cytokines as measured across platforms on the same clinical variables, we aimed to support the concurrent validity of MSD and Simoa. Though the absolute relationship between the measured cytokines and each external variable are still empirical questions currently under investigation (e.g., cognition and IL-6), we posited that the *relative* direction and size of the relationships should be consistent across the two platforms for the same cytokine marker to support convergence. Indeed, 95% confidence intervals of all associations examined overlapped between MSD and Simoa, though it is notable that these confidence intervals were fairly large (e.g., spanning $r<0.2$ to $r>0.6$ even for significant associations) and commonly crossed zero (non-significant). Both IL-6 and TNFα demonstrated consistent associations with external variables across platforms particularly in terms of direction, and neither MSD nor Simoa demonstrated consistently stronger or weaker associations overall. Regarding construct and ecological validities of the cytokine markers overall (i.e., degree to which each platform marker was associated with similar constructs and/or real-world status), IL-6 and TNFα demonstrated associations with external variables of interest in the expected directions, including positive associations with age and C-reactive protein, a clinically obtained marker of immune activation, and negative associations with

an executive functions task. On the other hand, perhaps less surprising given their relative discordance, IP-10 and, particularly, IL-10 demonstrated some differences across platforms. For IL-10, a generally anti-inflammatory cytokine, MSD demonstrated consistent negative associations with measures of cardiovascular disease and immune activation (C-reactive protein), whereas Simoa demonstrated small, but consistently positive associations with these factors. Similarly, regarding age, IP-10 (pro-inflammatory) demonstrated a significant negative association via MSD but a positive association via Simoa. Taken as a whole, our data largely supports the *relative* interpretation of cytokine concentrations when examining markers across platform techniques, though reliability of these relationships may still vary depending on analyte,

Our data are not without limitations. Most notably, though blood was drawn on the same day from the same participants, never underwent a freeze-thaw cycle, and was analyzed by the same lab technician, the analyses were not conducted on the same day (MSD aliquots were analyzed before Simoa aliquots). Though less likely to contribute significant measurement error, length of freeze time and human factors that can differ from day-to-day may have introduced additional noise not related to the platform quantifications themselves. Additionally, our data were collected in a fairly homogeneous patient sample of healthy, relatively well-educated, largely White older adults. Especially in the absence of disease, the range of cytokine concentrations may be limited (ceiling/floor effects) leading to smaller associations overall (including across platforms) simply due to limited psychometric power. Additionally, due to these limitations, we were not able to examine effects of other demographic factors such as race or low education on the cytokine markers, which may be of importance interpreting patient samples in diseased groups in the future. On the other hand, the uniformity of our clinical sample will help provide a baseline understanding not only of platform-specific nuances in cytokine measurement, but also the potential relative demographic influences for individuals similar to our sample.

Conclusions

Our study is among the first to closely characterize a common cytokine panel across MSD and Simoa platforms in a clinical sample. Consistent with previous studies examining comparability of other biofluid platforms, our data suggest that 1) platform selection matters, and 2) examination of *relative*, but not absolute values of cytokine concentrations may be the most prudent interpretation of these markers. However, the degree to which differences quantification approach impacts measured concentrations may differ by analyte.

While IL-6 and TNFα appeared relatively robust, IL-10 and IP-10 were much more sensitive to platform differences. As more diverse quantification approaches and studies emerge, more work is importantly needed to describe biofluid protein measurements across platform techniques, across time, and especially in diverse clinical samples; our conceptual findings will ultimately only be as meaningful as our measurement tools.

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