EDITORIAL COMMENT

Multiplexing and Beyond in Biobehavioral Research

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ABSTRACT

In contrast to traditional singleplex assays that provide values for only a single analyte in a single biological sample, multiplex assays are a time- and resource-efficient high-throughput approach that provides the opportunity to determine numerous analytes within a single- and small-sample volume. In this editorial on an article by Dorn et al. in this issue of *Psychosomatic Medicine*, we provide a brief description of the advantages and challenges related to multiplex assays. Although the use of multiplexing as a tool has been relatively limited in biobehavioral research, more recent studies are taking advantage of this technology to obtain deeper insight into regulatory patterns in health and disease states. Multiplex approaches range from several targets to global target profiling that importantly enable unbiased biomarker and pathway discovery.

Key words: multiplex, assays, biobehavioral.

"No man is an island." John Donne

In this issue of *Psychosomatic Medicine*, Dorn et al. (1) report novel and interesting cytokine patterns in a group of healthy adolescent girls. The group used a multiplex platform to simultaneously assess and model 13 different biomarkers representing adaptive T_H1 and T_H2 as well as innate immune pathway cytokines. In addition to the importance of the data revealing the innate variation of cytokines in healthy adolescent girls and their associations with mood and anxiety, the article also includes a valuable examination of two different data reduction approaches to simultaneously analyze multiple cytokine data (variable-centered [principal factor analysis] and person-centered data [latent profile analysis]), while discussing the merits and shortcomings of each. These statistical approaches may reduce biases related to multiple testing and statistical type 1 error (2). Although much attention in biomarker discovery has focused on discrete disease states, it can be argued that equivalent biomarkers of health are equally important to develop improved disease prevention strategies. The study by Dorn et al. (1) is significant in this regard.

We have come to understand that disease, particularly chronic disease, involves dysregulation of multiple pathways that often vary among afflicted individuals. As "No man is an island," so too no active biological compound exists unto its own in its respective milieu, and although the reporting of a single or very few biomarkers in a scientific study does possess value, that value would be markedly higher when information about the other numerous important integrated regulatory factors is added. For cytokines (such as interleukin 6), for example, and as noted by Kingsmore (3), the other important regulatory factors would include "the dynamic aggregate of multiple tissue-dependent agonist and antagonist cytokines, associated modifier proteins, receptors, and receptor antagonists." Multiplexing then is an efficient bioassay tool for measuring multiple analytes in a single biological sample. This is in contrast to traditional singleplex assays that provide measurements for only a single analyte in a single biological sample.

For many decades, enzyme-linked immunosorbent assays (ELISAs) have been a reliable workhorse of numerous laboratories and generally considered the criterion standard for protein assessment. Because ELISA platforms moved from singleplex to multiplex assays, singleplex ELISAs were used to test the accuracy and reproducibility of the multiplex platform ELISA (4). With advances in the reliability and validity of multiplexing platforms, the biomedical literature has in parallel shown a significant increase in multiplexing biomarker manuscripts across diverse types of biomedical research domains (Fig. 1). The literature reporting on multiple global "omics" strategies, including genomics, epigenomics, transcriptomics, proteomics, and metabolomics, has, too, grown significantly. This advancement to simultaneously assess numerous biomarkers within

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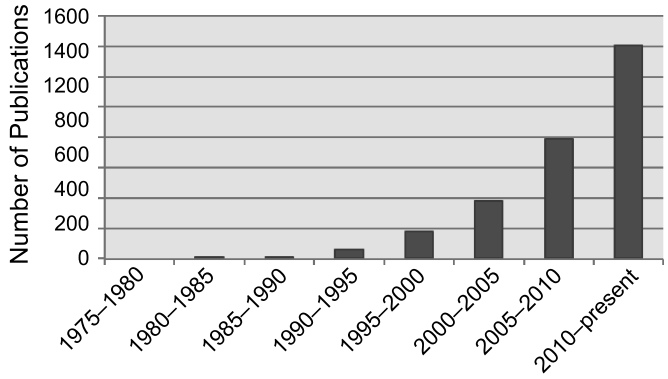

FIGURE 1. Number of publications appearing in PubMed by 5-year increments from 1975 to the present using the search terms "multiplexing," "assay," and "biomarker."

systems has obviously deepened our capacity to understand and model regulatory pathways and other phenomena across numerous complex biological systems in health and disease states.

It would be reasonable to say that although ELISAs made a relatively easy transition to multiplexing, other multiplexing approaches such as bead array assays were initially more problematic in terms of their validity and reliability. More recent performance evaluations of this platform, however, have been more favorable (5,6). Dorn et al. (1) used a Luminex Lincoplex multiplex immunoassay panel composed of a high-sensitivity 13-plex antibody bead array that permitted simultaneous measurement of multiple cytokines in a single, small volume blood sample. Other biobehavioral studies have used other multiplexing platforms, including the Meso Scale Discovery (Rockville, MD) ELISA platform (7–9).

Because the number of analytes to assess has increased in multiplexing, vendors have grouped them into "panels" for specific areas of interest. Analytes are typically grouped within themed panels and strategically paired based on compatibility of the chemistry as well as sample dilution needs. For example, cytokine, cancer genome, and kinome panels are often employed in biobehavioral research (1,10). Flow cytometry is regularly used in biobehavioral research to simultaneously quantify numerous cell surface and intracellular cytokines, not so much circulating levels. Depending on the vendor and platform, typical multiplex panels can provide simultaneous assessment of up to many dozens of biomarkers in a single sample of plasma, serum, urine, or saliva. Of course, the potential to expand the quantity of multiplexed analytes is as easy as combining values across a number of different panels. For example, cardiovascular disease researchers might combine proinflammatory, vascular injury, and chemokine panels to more thoroughly assess risk profile of a certain cardiovascular disease population or to assess the cardiovascular health-related benefits of a behavioral intervention. Arnold et al. (11), for example, combined several different cardiovascular and metabolic disease multiplex panels to yield a total of 190 assessed protein biomarkers that provided 80% accuracy to classify individuals into their correct depressive diagnoses.

What are pros and cons to employing multiplex assays in biobehavioral research? As far as concerns with multiplexing, many of these are the same that hold for singleplex assays, including the primary need for consistency and reliability of antibody quality across lots. This merits significant attention because antibody quality can be poor (12,13) and has been cited as a contributor to the current reproducibility crisis in biomedical science (14). Also, although there are generally good correlations among analyte levels across different multiplexing platforms, there are invariably significant differences in *absolute* levels (5). Thus and consistent with singleplex assays, it is important not to change multiplexing platforms midstream within a given study. Finally, there are noted limitations too to the proliferation of both diagnostic and prognostic protein biomarkers across many fields (15–18). Although these potential limitations are not a focus of this editorial, whether we are considering biomarkers for risk prediction or screening and diagnosis, limitations include issues such as validity, reliability, sensitivity, and specificity. In addition, the undermining effects of inherent high biological variability on the utility of commonly used biomarkers have been noted (17).

As exemplified in the study by Dorn et al. (1), there are both opportunities and challenges for data reduction and pattern recognition analysis that correspond to the increasing the number of biomarker outcomes. Their approach to the cytokine data included both variable-centered and person-centered methodologies; the former is to ascertain which cytokines to factor together, whereas the latter is to ascertain which study subjects to group together. The major cons related to global approaches include the lack of off-the-shelf systems biology data analysis packages and the challenge of integrating multiple data types. Such approaches are also not hypothesis driven but are often useful for hypothesis generation

and have shown great potential in disease modeling, diagnostics, and providing a greater understanding of systems, pathways, and their functionally dynamic interactions.

Although the use of multiplexing for proteins and neurohormones in biobehavioral research is increasing, their use has lagged far behind as compared with other fields. In addition, what constitutes "multiplexing" varies across fields of research and by orders of magnitude across platforms and substrates. In proteomics and metabolomics platforms, for example, which are less commonly used in biobehavioral research, the multiplexing scale increases approximately 10- to 1000-fold (19).

Future directions for biobehavioral research will include the increased use of multiplex platforms. Although it was historically challenging to find platforms that could adequately detect biomarkers at inherently low biological levels (i.e., below the lower limits of assay detection) and typically easier to assess levels that were elevated in disease states, newer higher-sensitivity platforms and kits do a better job at detecting levels in healthy individuals. Future studies will benefit the literature by reporting sensitivity ranges for the different analytes being assessed. Studies such as that by Dorn et al. (1) will continue to be undertaken on a larger and larger scale and the continued parallel development of statistical approaches will allow one to effectively address concomitant multivariate problems. Given the multicomponent, multipathway nature of human disease, global data sets, and powerful multivariate analysis procedures are in better alignment to understand the complexities of both health and disease. The integration of omics approaches such as global transcriptional profiling and transcription factor motif analysis in biobehavioral research has generated profound insights such as increased support for an immune system involvement in depression and fatigue (20). Genome-wide association studies, proteomics, metabolomics, RNA sequencing, and both exome and whole genome sequencing have been employed successfully in other fields, and efforts to integrate large data sets are rapidly evolving, for example, the Cancer Genome Atlas (21). In cancer research, for example, studies often integrate both genetic and environmentally influenced pathway data. Similarly, the increased use of multiple omics approaches and integration of those data sets may further enrich biobehavioral research. For example, approaches may include metabolomics of peripheral blood and data integration with observed gene, gene mutation, and protein expression patterns. Changes in gene expression patterns and metabolite concentration can be linked to behavioral changes and interpreted in a biological context. Future studies that include measurement of biomarkers of health may further advance the development of disease prevention strategies. The integration of these data types with clinical observations will improve our understanding of the molecular basis of disease as well as disease management and prevention.

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