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Blood Based Biomarkers

Update on ultrasensitive technologies to facilitate research on blood biomarkers for central nervous system disorders

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Abstract Most research on fluid biomarkers for central nervous system (CNS) disorders has so far been performed using cerebrospinal fluid (CSF) as the biomarker source. CSF has the advantage of being closer to the brain than serum or plasma with a relative enrichment of CNS-specific proteins that are present at very low concentrations in the blood and thus difficult to reliably quantify using standard immunochemical technologies. Recent technical breakthroughs in the field of ultrasensitive assays have started to change this. Here, we review the most established ultrasensitive quantitative technologies that are currently available to general biomarker laboratories and discuss their use in research on biomarkers for CNS disorders.

Keywords: Blood; Serum; Plasma; Biomarkers; Ultrasensitive; Central nervous system; Alzheimer's disease

1. Introduction

The goal of biomarker research is to provide objective tools that can be used for example in the clinical diagnostic workup, as inclusion criteria in clinical trials to enrich for patients with a certain type of pathology and to monitor treatment effects. In the search for biomarkers, it is assumed that the chance of finding good candidates is associated with the proximity to the origin of the disease. In diseases of the central nervous system (CNS), this would suggest that biopsies of the brain or spinal cord would be the ideal specimen to investigate. By its nature, however, this is almost without exception not possible due to the invasiveness of the procedure. Instead, analysis of cerebrospinal fluid (CSF) has been regarded a mirror of the metabolism or pathophysiological changes in the CNS. However, a lumbar puncture is needed to obtain CSF, and this technique is sometimes considered as an invasive procedure and might also give adverse events in the form of post-lumbar puncture headache. Therefore, a Holy Grail of biomarkers for CNS-related diseases would be to measure them in blood, which is more easily accessible.

A proteomic approach using mass spectrometry (MS) is often used in the search for biomarkers, and for small molecules such as amino acids and lipids, MS is also used in clinical routine settings [1]. The advantage of the method is that it directly measures the molecule of interest but on the downside are low throughput and an inability to measure intact larger proteins compared to immunoassays. As the name implies, immunoassays use antibodies to quantify a substance in a sample. A common technique is the sandwich enzyme-linked immunosorbent assay (ELISA) in which most often the analyte is captured between two antibodies in a sandwich-like complex and one of the antibodies carry a signal generator, that is, an enzyme which converts a substrate into a detectable form (colored, fluorescent, or luminescent products) which in combination with a calibrator curve allows for quantification of the analyte of interest. ELISA is a theme with many variations

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such as in the choice of signal generator where the enzyme can be exchanged with for example a fluorophore or a DNA-based system. In a recently described technology, ELISA has been combined with MS-based quantification of the enzymatic products. The technology is called enzyme-linked immuno mass spectrometric assay and may provide increased analytical sensitivity, as compared to regular ELISA, by reducing the background [2]. Immunochemical assays may also be multiplexed in different ELISA-like formats. Such biomarker panels are frequently examined in the current biomarker literature with focus on CNS disorders [3,4]. A downside with multiplexing, however, is that it may be hard to optimize the analytical conditions for several antigen-antibody interactions, especially if their concentrations or biochemical characteristics substantially differ, as compared to optimizing assays that focus on the accurate measurement of a single analyte.

There are several issues, both biological and technical, with the search for CNS-related biomarkers in blood. First, a biomarker that has its origin in the CNS has to cross the blood-brain barrier to be detected in the periphery and if the concentration is low in CSF then it will be even lower in the blood due to the blood:CSF volume ratio causing a substantial dilution. Second, if the biomarker is not specific for the CNS but also produced in the periphery, then the contribution from CNS will potentially drown in the high biological background caused by non-CNS sources (a good tool to assess the risk for this is the publicly available web-based Human Protein Atlas, http://www.proteinatlas.org/, which presents protein expression in 44 different human tissues of close to 20,000 proteins [5]). Third, the huge amount of other proteins in blood (e.g., albumin, immunoglobulins) introduces analytical challenges due to possible interference. Fourth, heterophilic antibodies may be present in blood at high concentrations that may give interference in sandwich immunoassays. Fifth, the analyte of interest may undergo proteolytic degradation by various proteases in plasma.

The technical issues are mainly a question of sensitivity and antibody specificity. Ideally, the enzyme reaction that is the final step in an ELISA should be able to increase the sensitivity by simply extending the reaction time. However, the substrates used are inherently unstable and therefore produce signal even in the absence of enzyme. This leads to a technical background signal that can mask the signal caused by the sandwich complex making quantification difficult at low concentrations. The ability of the sandwich complex to correctly represent the concentration of the biomarker in a sample strongly depends on the quality of the antibodies used. If the antibodies cross-react with other substances then a signal can be measured even in the absence of the biomarker toward which the assay was developed. As the blood is much denser in protein content than is CSF the risk for this is higher in the former.

The biological issues are refractory but the technical ones can be addressed. The production of antibodies is sometimes described as science, art, and magic, and by its nature, some luck is needed to produce high-quality monoclonal antibodies. For the problem with background, however, there are solutions and this is what the ultrasensitive technologies have found different ways of solving.

In this overview article, we focus on describing and discussing currently available ultrasensitive technologies that may be useful for measuring CNS-specific or enriched proteins at low concentrations in the bloodstream.

2. Platforms

Most of the different methods described below build on the sandwich principle for antibody-based quantification discussed above and differ primarily in the method for detection. A brief description of each method is given, and further details can be found in the cited publications. In general, the technologies used are proprietary with the platforms available from only one company. Examples where the platforms have been used in the field of CNS disorders are also given.

2.1. Single-molecule array

Single-molecule array (Simoa) is a digital ELISA that has been invented and commercialized by Quanterix (www.quanterix.com) [6]. After the formation of the sandwich complex on magnetic microbeads, these are transferred, in substrate solution, to an array of 300,000 micro wells. These wells can accommodate only one bead each and, after the addition of a fluorogenic substrate for the enzyme with which the detection antibody is labeled, an oil film is then applied to seal the wells confining the reaction volume to 50 fL. This small volume allows for a readable signal to be detected even if only one sandwich complex is present on the bead. As reporters, the enzyme β-galactosidase and the substrate resorufin-β-D-galactopyranoside are used and the wells having a fluorescent signal are counted as are all the wells containing a bead. The ratio between these counts provides the output average enzyme per bead (AEB) number. When the AEB is low (<0.1), Poisson statistics shows that either a bead has only one or none sandwich complex on its surface, hence the name digital ELISA. When the AEB signal gets higher, increasing the probability of more than one complex per bead, there is a transition to the utilization of also the light intensity which allows for a usable AEB even at signals >0.1. The algorithm for the transition is implemented in the software that comes with the fully automated Simoa instrument to which samples and calibrators can be fed either using a 96-well microtiter plate or vials.

2.2. Single-molecule counting

In the single-molecule counting (SMC) platform, the sandwich complexes, originating either from beads or plates, are broken up and only the fluorescently labeled (Alexa Fluor) detection antibody is drawn into a capillary tube and counted one by one as they pass a laser beam that excites the fluorophore. A digital event is counted if the fluorescence reaches above the threshold of the background. At higher concentrations, it is difficult to separate all events and a switch is made to use the total sum of all emitted photons as readout for the