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REVIEW

Progress and promise of pharmacodynamic biomarkers: novel strategies and assay considerations in drug development

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ABSTRACT

Pharmacodynamic (PD) biomarkers provide crucial insights into a drug's mechanism of action (MoA) and efficacy by measuring its effects on biological targets within an organism. PD biomarkers can be proximal (e.g. receptor occupancy, enzyme inhibition) or distal (e.g. downstream pathway modulation) to the biological target. In drug development, PD biomarkers are essential for monitoring patient response, assessing therapeutic efficacy, optimizing dosage strategies, and streamlining the drug development process by informing go/no-go decisions. In personalized medicine, PD biomarkers enable tailored treatments based on individual responses, enhancing both effectiveness and safety. Sound bioanalytical strategies and rigorous assay validation practices are key for successful integration of PD biomarkers into clinical trials. This paper outlines the bioanalytical and assay considerations for developing and validating informative PD biomarker assays and their use in drug development.

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PD biomarker; bioanalysis; mechanism of action; assay validation; antibodies; context of use; fit-for-purpose

1. Introduction

Pharmacodynamic (PD) biomarkers are molecular indicators that measure the effects of a drug within an organism [1]. These biomarkers are categorized into three main types commonly used to highlight various aspects during drug development: target engagement, pathway modulation, and disease modulation (Figure 1). PD biomarkers may act proximally to the drug target such as in cases of receptor occupancy, soluble target engagement, or enzyme inhibition, or distally where their effects are observed through the modulation of downstream pathways or as disease indicators.

In drug development, PD biomarkers play a pivotal role in proving hypotheses regarding the mechanism of action (MoA), demonstrating that a drug is interacting with its intended target as expected and exerting its biochemical and physiological effects (proof-of-mechanism) and could also support regulatory approval [2,3].

Reliable quantitative PD biomarkers are instrumental for optimizing dosing by elucidating the relationship between exposure and biological response (PK/PD). This understanding enables drug developers to determine the ideal dosage and schedule that maximizes efficacy and convenience while minimizing side effects. Furthermore, PD biomarkers assist in assessing efficacy by confirming whether the drug induces the desired biological effect (proof-of-concept), which is essential during early-stage clinical trials. Beyond utility related to PK/PD evaluation and dose optimization, PD biomarkers are valuable for personalized medicine by enabling the customization of drug therapies based on an individual's specific biological responses. This customization may lead to safer and more effective treatments.

Additional applications of PD biomarkers include biosimilar development and expansion of proven drugs into new indications. In biosimilar development, PD biomarkers are used to demonstrate similarity to the original product, rather than independently establishing the safety and effectiveness of the biosimilar, to support new drug approvals. When expanding into new patient populations, PD biomarkers help demonstrate whether the same exposure–response relationship holds true in the new population and justifies dosing in that population. If the PD biomarker data related to pathway intervention shows maximal response (i.e., saturated) without any clinical benefit in early trials, a no-go decision can be achieved quickly to rule out a clinical hypothesis yielding significant resource and cost savings. Therefore, the promise of biomarkers is to reduce the overall time to bring a new drug to market and generate data for more informed decision-making along the entire drug development pathway. Between 2015 and 2020, biomarkers were used in over 50% of drug approvals by FDA and EMA [4].

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Article highlights

Introduction
- PD biomarkers validate mechanism of action (MoA) and target engagement, and inform on physiological effects.
- They support dose optimization, accelerate decisions, and enhance trial efficiency.

Preclinical Decision Framework
- Assay development starts early, ideally after target validation, and evolves throughout the clinical program.
- Lifecycle includes discovery, development, validation, and implementation aligned with context of use (COU) and transitions to clinical development.

Early Drug Response Monitoring
- PD biomarkers help characterize onset, magnitude, and duration of drug effects, and guide dose and schedule optimization.
- Case studies illustrate successful use early clinical development to optimize dosing for confirmatory trials.

Assay Development, Optimization & Validation
- Platform Selection: Based on COU, biomarker properties, and assay requirements.
- Method Optimization: Uses reference materials and statistical design (e.g., DoE) to refine conditions.
- Solutions for bioanalytical challenges include technological innovation, robust validation, and strategic planning.
- Validation assesses parameters such as accuracy, precision, sensitivity, specificity, dynamic range, stability and for some technologies, parallelism.
- Fit-for-Purpose (FFP): Validation rigor matches the biomarker's COU.

Integration into Clinical Trials
- Begin biomarker planning in preclinical phase using COU/FFP principles.
- Use multimodal approaches and adaptive designs to link biomarkers with outcomes.
- Leverage emerging technologies for deeper insights.

Recent Advances, Emerging Trends and Alternative Approaches
- AI enables mining of clinical datasets for biomarker discovery.
- Liquid biopsies offer accessible alternatives to tissue biopsies.
- Advanced platforms and approaches (e.g., high-dimensional flow cytometry, mass cytometry (CyTOF), high multiplex assays and imaging, multi-omics, spatial transcriptomics, mass spectrometry imaging and thermal shift assays) can enhance biomarker assessment.

Conclusion
- PD biomarkers are essential tools for understanding drug effects and guiding development.
- Success depends on robust assay design, validation, and implementation into clinical strategy.
- Technological progress continues to expand biomarker applications

The discovery and validation of novel PD biomarkers have been further accelerated with the use of new multiplexing and multi-omics technologies. Higher-dimensional techniques such as next generation sequencing (NGS), immunohistochemistry (IHC) multiplexing, imaging (radio/spatial) and transcriptomics have not only provided insights into the identification of novel biomarkers but also have the potential to improve the understanding of molecular interactions. Recent technological advances in reagents and detection platforms have improved their sensitivity and dynamic range. Given the critical role of PD biomarkers in drug development, this perspective paper outlines key bioanalytical and assay considerations for developing and validating biomarker assays and their use in clinical development [5]. PD biomarkers that may be used for individual patient management fall outside the scope of this review.

2. Preclinical decision framework

Implementation of PD biomarker strategies in early drug discovery and development yields crucial mechanistic insights previously unattainable through conventional approaches, especially in understanding dose–response relationships in preclinical and early clinical studies [6]. Incorporating PD biomarkers into preclinical development should begin concurrently with initial drug development, immediately following target validation. Early integration facilitates efficient lead-compound screening and selection processes during *in vivo* evaluation. The preclinical stage also allows for biomarker identification using targeted and omics-based technologies, and assessment in both peripheral and tissue compartments, enabling drug developers to identify markers with robust responses to advance into clinical development.

Early PD biomarkers frequently serve a dual purpose, functioning as both target engagement indicators and downstream pathway markers [7]. Early characterization of temporal dynamics between site-of-action responses and circulating biomarker levels informs optimal monitoring and dosing strategies. Understanding these relationships enables the development of sampling schedules that capture meaningful biological changes while minimizing animal and patient burden during preclinical and clinical studies. Such understanding proves particularly valuable in chronic dosing scenarios, where long-term monitoring must balance scientific rigor with practical feasibility [8].

Successful programs typically employ multiple biomarker approaches such as direct binding assays supplemented with functional readouts, downstream pathway analyses, or feedback loop assessments. These approaches can be adapted to various formats and readouts, including biochemical, *in vitro* cell-based, and *in vivo* sample-based platforms, thus ensuring continuity from initial *in vitro*/*ex vivo* potency testing to *in vivo* PD assessment.

Establishing clear decision frameworks based on early PD biomarker data is critical for driving the success of early drug discovery and development programs. Such frameworks enable organizations to make go/no-go decisions and to allocate resources more effectively. Project teams must consider several key factors in preclinical PD biomarker development:

(1) Biological evidence demonstrating specificity and mechanism relevance
(2) Analytical feasibility including throughput and technology transfer
(3) Translatability of the assay technology and biology across preclinical species and to clinical settings
(4) Correlation between site of action responses (where measurable), circulating/peripheral or imaging biomarker changes, and proposed clinical efficacy endpoints

Programs should define the limits of technical and biological variability in biomarker measurements, while maintaining rigorous standards for demonstrating biological effect [9]. Project teams should also clearly state assumptions related to correlation and translatability. For cases where no specific downstream pathway response is evident, additional omics profiling may be helpful.

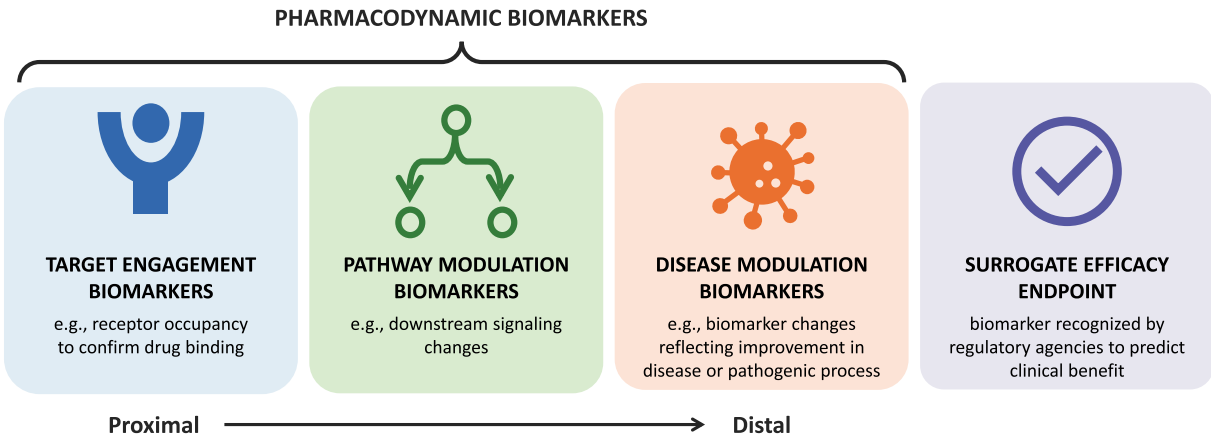

Figure 1. Pharmacodynamic biomarkers as informative indicators during drug development. Pharmacodynamic (PD) biomarkers can be used to assess target engagement and pharmacological response during drug development. Responses to drug effects may include modulation of proximal or distal biological pathways. PD biomarkers can also serve as surrogate efficacy endpoints to support efficacy outcome if a direct connection to clinical outcome is established.

3. Assay development, optimization and validation

The lifecycle of a biomarker assay encompasses the entire drug development process, from initial assay design and development to validation, routine use, and eventual retirement or modification, ensuring the continued suitability and performance of the assay. A critical first step in planning PD biomarker assessments is understanding the biological context of the biomarker and its role in disease pathophysiology. Selecting the most appropriate technology platform to measure the PD biomarker depends on the sensitivity required, the characteristic to be analyzed (e.g., protein concentration, isoform, functional activity, lipid concentrations, sterol concentrations, etc.) and the biological matrix (e.g., plasma, serum, CSF, urine, tissue biopsy). Additionally, determining whether the analyte is soluble, secreted, or best evaluated using cell-based or tissue-based assays is essential. Establishing a clearly defined context of use (COU) serves as a foundational step for technology selection and the subsequent development and validation of the biomarker assay.

Figure 2 illustrates the iterative evolution of a PD biomarker assay – from understanding biomarker biology and defining the COU to initial assay feasibility and refinement into a final validated assay – while incorporating new information and technological advancements.

3.1. Choice of technology platform

Analyzing PD biomarkers often requires specialized methods that are carefully tailored to both the biomarker types and research objectives. The criteria and considerations for platform choice include assay sensitivity, availability, ability to multiplex, physicochemical properties of the analyte or complex, as well as practical factors such as logistical feasibility, throughput, and turnaround time. Highly sensitive platforms, such as mass spectrometry (MS) and ligand-binding assays (LBA), are frequently used; however, each has its own set of limitations, which in turn shape the choice of assay format. For single-cell analysis, several flow cytometric assay formats enable receptor occupancy measurement of monoclonal antibody therapeutics targeting cell surface markers, informing PK/PD modeling and early clinical dose selection [10–12]. Some commonly used technologies and their applications are summarized in Table 1. These technologies are compared in terms of sample matrix, calibrator material, type of measurement, advantages, and challenges.

Beyond these common platforms, additional approaches can be suitable to investigate different biomarker characteristics.

- *Western blotting*: Used for monitoring changes in protein expression level or isoform proportions post-treatment [42,43].
- *Functional Activity Assays*: ELISpot and enzyme activity assays are used depending on the drug's MoA and anticipated biological effects. ELISpot can quantify cytokine secretion from immune cells and enumerate antigen-specific immune cells for vaccine efficacy evaluations, while enzymatic activity assays (either MS-based or fluorometric plate-based) are important for determining functional gene replacement success in gene therapy [44–47].

For therapeutic programs involving biomarkers requiring detailed spatial analysis, conventional platforms may lack the resolution to support tissue- or cell-level PK/PD elucidation, such as those relevant to xRNA and antisense oligonucleotide (ASO) therapies. Imaging technologies, such as Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI), and Computed Tomography (CT), provide near real-time information on drug distribution, target engagement, and early pharmacological responses at the tissue and cell level [48–50]. These techniques rely on fluorescent- or radio-labeled probes and enable visual assessments of anatomic regions-of-interest for diagnostics, disease monitoring, or therapeutic response evaluations. Spatial PD biomarker information can also be obtained semi-quantitatively using IHC or *in situ* hybridization RNA analysis platforms like RNAscope [51]. However, these approaches require tissue biopsies, making them less ideal for capturing temporal PD data in clinical trials.

Biomarker and bioanalytical teams should evaluate the most suitable technology platform based on the defined COU. Testing multiple technology platforms during assay

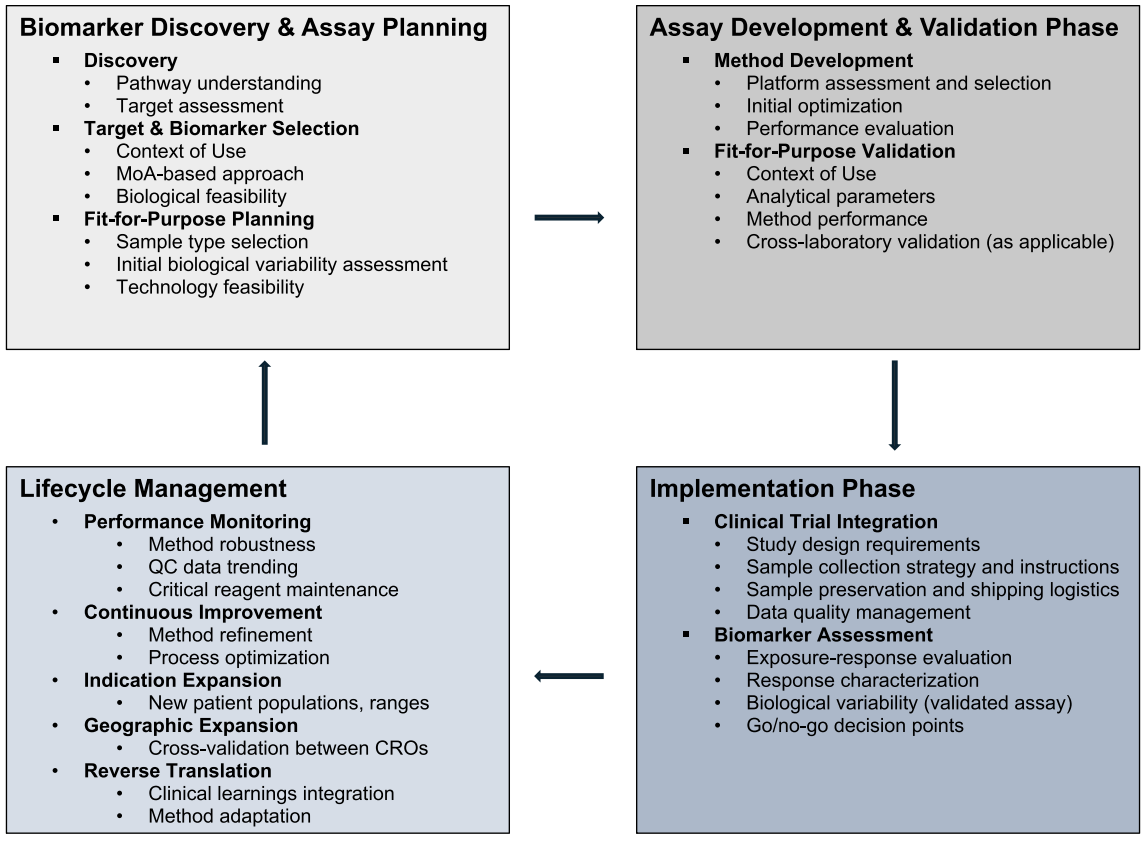

Figure 2. PD biomarker assay lifecycle framework. The framework includes four phases of biomarker assay development that allows organizations to effectively employ PD biomarkers in their drug development programs. The conception of a PD biomarker assay begins early during drug discovery and undergoes iterative refinement throughout the duration of the clinical program as the context of use of the biomarker changes. MoA, mechanism of action; QC, quality control; CROs, contract research organizations.

development may be necessary to determine the most appropriate choice for PD biomarker assessments. Assay parameters must be characterized thoroughly to ensure reliability.

3.2. Method development and optimization

Assay development is guided by the availability of reference materials and the characteristics of the biological control matrix. Reagent availability plays a critical role in shaping the validation approaches for assays across technologies. For instance, flow cytometry lacks true cellular reference materials, preventing the use of calibration standards and quantification via a standard curve [27]. In contrast, small-molecule biomarker analysis via mass spectrometry often uses stable isotope-labeled analogs as calibrators. Stable isotope-labeled analogs behave comparably to the native analyte and are distinguished by the mass difference between the two. This mass difference allows the use of native matrix for calibrator preparation (absolute accuracy). Conversely, ligand-binding assays rely on recombinant material as calibration standards, which may not entirely reflect the intrinsic properties of the endogenous analyte (relative accuracy).

The application of artificial intelligence (AI) and machine learning (ML) tools (e.g., Gemini, Copilot) can streamline method development by summarizing relevant literature and creating preliminary assay frameworks. Following feasibility assessments with the chosen technologies, assays are optimized by adjusting reagent sources, configurations, and various analytical parameters. Throughout this iterative process, every adjustment remains closely aligned with the biomarker's COU.

Design of Experiments (DoE) is a robust statistical method for optimizing assay conditions and parameters by systematically varying multiple factors and analyzing their impact on the assay outcome. This approach enhances efficiency, reliability, and effectiveness [52,53].

Examples of DoE applications include:

- *Cell-based assays*: Optimization of cell culture conditions, buffer characteristics, and incubation times [54].
- *Ligand binding assays*: Refinement of intra-plate parameters such as coating, detection antibody concentration, and streptavidin – HRP concentrations, as well as inter-plate parameters like incubation durations [55].
- *PCR assays*: Adjustment of annealing temperature and reaction time [56].
- *LC-MS/MS methods*: Optimization of liquid chromatography and mass spectrometry parameters for bioanalytical quantitation [57].

The DoE approach has been instrumental in accelerating the development of robust and reproducible assays across these examples, proving valuable in assay optimization efforts.

Table 1. Most common technology platforms for PD biomarker assessments.

	Mass spectrometry	Ligand binding assays	Genomic techniques	Flow cytometry	IHC/IF/Image analysis
Sample matrix	Biofluid, cells and tissue	Biofluid, cells and tissue	Biofluid, cells and tissue	Biofluid, cells, tissue	Cells and tissue
Type of measurement	Absolute or relative quantitation	Relative quantitation	Relative quantitation (qPCR); absolute quantitation (dPCR, NGS, ctDNA); Relative expression (RTqPCR, RNAseq, Nanostring, etc.)	Qualitative, semi/quasi-quantitation or relative quantitation	Qualitative or semi-quantitation (conventional) Relative quantitation (digital)
Calibrator material	Calibrators for small molecule biomarkers is chemically identical to endogenous material; may use stable isotope analogs to allow use of native matrix. Calibrators for large molecule biomarkers or small molecules with post-translational modifications may not be representative of endogenous material.	Calibrator may not be representative of endogenous material	Calibrators include the target sequence (surrogate reference standards, plasmids, gBlocks, synthetic fragments); standard curve used in qPCR	Absence of well-characterized, suitable, representative cellular reference material for most assays	Standard reference material (Purified analytes conjugated to a solid phase that are traceable to NIST SRM 1934) available in limited cases (e.g., HER2, PD-L1, Ki-67, ER)
Advantages	Direct measurement of analyte of interest; wide dynamic range; high-sensitivity for low abundance biomarkers; high specificity and selectivity; multiplexing; use of stable isotope internal standards to correct for sample preparation and ionization differences; native matrix or surrogate matrix – for some protein/peptide biomarkers, may use matrix from a different species; amenable to automation; extraction reduces affect of biotherapeutic binding interference	Widely available technology and expertise and relatively easy to execute; some immunoassay platforms are more sensitive than others, e.g., Simoa, ELLA, MSD vs. traditional ELISA format; potential for automaton (e.g., Gyrolab, ELLA); multiplexing; some platforms offer wide dynamic range; high throughput; low sample volume needed	High-sensitivity, high specificity, sample stability; high throughput capabilities and multiplexing reactions for evaluating multiple biomarkers from a single sample	High-sensitivity; multiparameter analysis allows for simultaneous functional and phenotypic characterization of individual cells from distinct cell populations in a single sample; surface, intracellular or intra-nuclear targets can be assessed; high resolution and sample throughput (cells/uL); can detect, enumerate and characterize rare cellular events	Provides general cell morphology, tissue architecture and positional information; integration of fluorescent probes enhances multiplexing and allows for high-resolution imaging (exploratory); opportunity to use digital pathology imaging tools and AI to enable standardized analysis
Challenges and limitations	Specialized instrumentation and expertise; high cost; requires ionizable groups; limitation in analysis of long oligonucleotides; matrix effects; sample volumes may be restrictive especially in preclinical animal models and pediatrics; analysis time may be rate limiting	Indirect measurement of analyte of interest; lower cost than mass spectrometry; availability of antibodies for detection/capture; potentially long lead time for critical reagents; assay reproducibility affected by reagent constraints, lot-to-lot variability; assay specificity, matrix effects and hook effect; less amenable for small molecule analytes	Specialized space, instrumentation and expertise; special precautions required to prevent sample contamination with nucleases; variability due to extraction; PCR inhibition; choosing appropriate reference genes for normalization; tissue sample stability; on-treatment sample availability	Specialized instrumentation and expertise; QC material availability, sample stability and cell viability; standardization is critical to ensure reproducibility and reliability of results over time (inter-instrument, inter-laboratory, methodology, data analysis); single-cell suspension requirement; sample processing and/or data analysis can be complex and extended	Specialized expertise, more specialized instruments for IF; requirement for repeated sampling limits use for investigating temporal changes; limitations on sample quantity and quality; lower throughput; proper tissue preparation is critical for preserving antigen integrity and enabling accessibility; antibody selection; high background/nonspecific staining possible; antibody cross-reactivity; traditional methods lead to high variability due to subjective data analysis; batch variation
Common applications	Small molecule metabolite panels, protein and lipid identification and quantitation; peptide mapping or de novo sequencing; post-translational modification characterization and quantitation	Protein biomarker quantitation; chemokine/cytokine panels	Biomarker expression levels (mRNA), RNA/miRNA profiling, copy number analysis; measurable (or minimal) residual disease (MRD) via NGS or PCR, mutation detection	Immune cell subset phenotyping, receptor occupancy, CAR-T persistence, antigen-specific T cell (tetramer) enumeration; intracellular cytokine staining; cell proliferation; phosphoprotein analysis; MRD	Tumor microenvironment characterization; detection/quantitation of protein or target in disease tissue; cellular interactions, protein localization and distribution; gene expression signatures

(*Continued*)

Table 1. (Continued).

	Mass spectrometry	Ligand binding assays	Genomic techniques	Flow cytometry	IHC/IF/Image analysis
Example clinical trial applications	• Plasma clofazimine PK analysis in MDR-TB patients • Progesterone quantification in postmenopausal women using LC-MS • Plasma pTau217 analysis in patients with early symptomatic Alzheimer's disease treated with donanemab	• ELISA quantification of VRC07-523LS in HIV bnAb trial • MSD cytokine profiling in peritoneal fluid for ovarian cancer immunotherapy • NfL measurement in Multiple Sclerosis and Alzheimer's disease	• qPCR tracking of BCR-ABL1 in CML (IRIS Phase III trial) • ddPCR-based plasma EGFR ctDNA kinetics in NSCLC and NGS-defined MRD in AML	• Vaccine applications • Safety, Activity, and Immune Correlates of Anti – PD-1 Antibody in Cancer • TIM-3 receptor occupancy in a solid tumor immunotherapy trial • MDSC and Treg monitoring in β-blocker + PD-1 melanoma trial • PD-1 receptor occupancy on circulating T cells in solid tumors	• Digital image analysis of multiplex fluorescence IHC in colorectal cancer recognizes the prognostic value of CDX2 and its negative correlation with SOX2 • Pharmacodynamic tissue studies, EGFR and downstream signaling components in tumor and skin • TIM-3 receptor occupancy in a solid tumor immunotherapy trial • PD-1 receptor occupancy on circulating T cells in solid tumors
References	[9,13–16]	[13,17–20]	[21–26]	[13, 27–32]	[30, 32–41]

3.3. Bioanalytical challenges and strategies

A significant challenge in developing and optimizing biomarker assays is the availability of suitable reference materials for quantitative measurements that are representative of the endogenous molecule. For example, for protein biomarkers, a recombinant protein may not fully represent the heterogenous endogenous analyte [58,59]. Equally important is the alignment of the biomarker dynamic range with the assay dynamic range [9]. A narrow assay dynamic range compared to the biomarker range will limit the utility of the assay.

Additional challenges include: sample heterogeneity, particularly in cell-based or tissue-based material; cross-reactivity and biological interference which may affect specificity of the assays; and biomarker stability – many PD biomarkers (e.g., lipids, proteins, RNA, metabolites, cells) are sensitive to collection, processing and storage conditions and/or are unstable in biological fluids [60,61].

Key recommendations include:

- *Reference materials*: Reference materials should be well-characterized when feasible. For small molecule biomarkers, use of stable isotope-labeled analog reference materials which are chemically identical to the biomarker and differ in mass from the endogenous analyte allows quantitation in native matrix. For protein biomarkers, characterize material by multiple analytical techniques, e.g., Octet, SDS-Page, HPLC, Aggregation, LC-MS or post-translational modifications (PTM) [59]. For surrogate analyte LC-MS/MS methods using peptide level calibration, full characterization of the synthetic peptides including amino acid analysis is recommended. Novel PD biomarkers require planning for reagent generation. There are ongoing initiatives to generate suitable reference materials led by NIST in the United States, the TATAA Biocenter in Sweden, and the European Commission's Joint Research Centre (JRC)'s Institute for Reference Materials and Measurements (IRMM) in Belgium.
- *Biomarker dynamic range vs assay dynamic range*: Advanced, high-sensitivity analytical techniques such as two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS), multiplexed biomarker panels, and highly sensitive immunoassays, allow detection of low-abundance biomarkers in sample matrices [62–68]. Methods such as affinity capture LC-MS, are also advantageous for enriching biomarkers of interest, thereby enhancing the sensitivity of targeted biomarkers, and potentially reducing matrix interference [69–71]. Recent advancements in digital PCR (dPCR) offer high sensitivity by leveraging partitioning and absolute quantification to measure trace amounts of nucleic acids [72].
- *Sample heterogeneity*: Follow recommendations for the quantitation of small and large molecule biomarkers in cell-based or tissue-based material as described in [60].
- *Cross-reactivity and biological interference*: Consider the interferences that are most likely to confound the

intended biomarker assay result. Assess interference by preparing QCs in the samples known to contain the interference [9,73]. Implement high-specificity assays, for example, assays based on monoclonal antibodies for LBA and hybrid-LBA-LC-MS/MS assays, to help minimize cross-reactivity and ensure accurate quantification. Implement orthogonal approaches to confirm the specificity of these monoclonal antibodies. The orthogonal approaches may include Western blotting (WB) or WB coupled with immunoprecipitation (IP) [42].

- *Biomarker stability*: Prepare stringent protocols for sample collection, storage, and transport as described in [61], to preserve the integrity of the biomarker. Sample stabilization and preservation methods may be feasible for some applications [74–79].

Biological variability will also influence PD biomarker measurement and data interpretation. Strategies such as collecting samples at the same time each day can reduce diurnal variation in concentrations [9,80]. In addition, implementation of a robust QC strategy can inform on individual assay performance acceptance as well as allow for the monitoring of assay performance over time, especially critical in a clinical trial of long duration.

Addressing the challenges associated with PD biomarker assessment may require a multifaceted approach that combines technological innovations, methodological improvements and strategic planning throughout the drug development process.

3.4. Validation processes and regulatory considerations

Regardless of the technology selected, core assay parameters should be assessed to effectively characterize the analytical method [13]. These parameters typically include accuracy (absolute or relative), reproducibility or precision, sensitivity, specificity, assay range, and stability. However, certain evaluations may be more important to some technologies than others. For instance, cell-based flow cytometric assays, which are quasi-quantitative, do not routinely assess accuracy as they lack reference standards and calibration curves. When regulatory requirements mandate accuracy in fit-for-purpose (FFP) validation, alternative methods must be used to address the lack of reference standard or calibration [27]. In the case of ligand binding assays, a critical parameter is parallelism; however, this assessment may not be applicable to other platforms.

Accurate and reliable measurement methods are critical for PD biomarker assessments. Because the biology of each biomarker is unique, and its intended use can impact various decisions – such as regulatory approval, dose justification, or internal decision-making – regulatory agencies and industry groups have sought to provide frameworks for assay validation. It is recognized that one-size-fits-all approaches are not feasible, as biomarker assays differ significantly from PK assays [13,81].

Jean Lee et al. introduced the FFP paradigm for biomarker method validation, emphasizing that the stringency of assay validation processes should align with the intended use of assay results [13]. While the 2018 FDA Bioanalytical Method Validation (BMV) and ICH M10 guidance documents provide clear frameworks for validating PK assays, biomarker assays require distinct approaches, a position that was reinforced in the recently published 2025 FDA guidance for bioanalytical method validation for biomarkers (BMVB). Despite these regulatory updates, industry groups continue to acknowledge the distinct requirements of PK assays versus biomarker assays given the significant differences in the analytes being measured (drug vs. biomarker), biomarker biology, and COU. As a result, biomarker assay validation should consistently adhere to the FFP paradigm. Scientists may refer to regulatory guidelines for initial direction, but parameters, specifications, and acceptance criteria should be adapted or excluded to appropriately support the biomarker's COU.

To further advance biomarker assay validation, the FDA and the Critical Path Institute collaborated on an initiative that culminated in a consensus document authored by scientific and regulatory stakeholders. This seminal white paper, written by Piccoli, Sauer, and the Evidentiary Considerations Writing Group (2019), provides a framework for validating assays used for fluid biomarkers, especially LBA and mass spectrometry assays. The paper builds upon the FFP framework and extensively addresses pre-analytical considerations and analytical parameters for biomarker assay validation based on COU principles. For detailed validation approaches applicable to PD biomarkers at different stages of development, refer to Table 1 in the 2019 "Points to Consider" paper [81]. Appropriate approaches for analytical validation of biomarker assays have also been described by the European Bioanalytical Forum (EBF) [82], the American Association for Pharmaceutical Scientists (AAPS) [83,84], and others [85]. Note that with wide adoption of the FFP validation paradigm and use of the term "qualification" as a description of Drug Development Tools recognized by the FDA, "qualification" is no longer a preferred way to describe the rigor of biomarker validation approaches.

3.5. Recommendations to guide biomarker assay validation for different technologies

Since the introduction of the FFP paradigm by Lee et al. in 2006, numerous recommendations and best practice white papers have been published to foster industry-wide consensus on assay validation requirements for different technologies [13]. Key highlights include:

- *Ligand Binding Assays and Mass Spectrometry*: Recommendations for LBA validation are predominantly addressed in Piccoli and Sauer, which also acknowledges mass spectrometry-based assays [81]. A second publication has since outlined more in-depth considerations and recommendations for assays using mass spectrometry-based assay validation [9].
- *Quantitative and Digital PCR*: Several working groups have collaborated to publish a series of white papers addressing best practices for PCR assays [22,56,86].
- *Flow Cytometry*: A wealth of literature has been dedicated to flow cytometric assay validation. Best practice

documents cover various topics, including instrumentation, receptor occupancy, CAR-T cell analysis, rare event analysis, method validation protocols, and method transfer [12,87–93]. A formal guideline, CLSI H62, focused specifically on flow cytometric assay validation, was published in 2021 [27].

- *Western Blot*: An AAPS working group recently published a best practices white paper on validation of western blot assays [42].
- *Enzyme Activity Assays*: A cross-industry group has published a best practices white paper for the development and validation of enzyme activity assays [45].
- *Immunohistochemistry (IHC) and Multiplex IHC/IF*: Regulatory, CLSI and CAP guidelines exist for analytical validation of IHC assays for clinical use [94,95]. These and best practices documents can guide method validation and imaging analysis for multiplex IHC/IF assays used in earlier development [96,97].

As biomarker assay technologies continue to evolve, these recommendations and guidelines serve as a vital resource for ensuring robust and reliable validation processes aligned with COU principles.

4. Integration into clinical trials

Integrating PD biomarkers into clinical trials can significantly enhance the understanding of a drug's effects and MoA throughout the development process [85,98,99]. The implementation of PD biomarkers enables rapid determination of the efficacious dose and supports precise dose optimization based on individual patient responses. Recent clinical studies demonstrate that PD biomarker-guided decision making has improved treatment outcomes and patient care [100]. To successfully incorporate PD biomarkers into clinical trials, the following steps should be considered:

4.1. Start early

PD biomarker assay development and biomarker validation should begin during the preclinical phase and continue through early clinical development to ensure their reliability and relevance. This early-stage validation provides a foundation for clinical biomarker planning. Preclinical studies allow for biomarker exploration and identification, enabling differentiation between biomarkers critical for decision-making and those suited for exploratory purposes during clinical trials.

4.2. Apply COU principles

The optimal approach involves clearly defining the COU, specifying how the biomarker will be used and its role in decision-making. This begins with basic validation of exploratory biomarkers and becomes progressively more rigorous as the biomarker's clinical importance grows, ensuring efficient resource allocation. Key aspects include:

- Alignment with Regulatory Standards: Validation processes should adhere to regulatory requirements, facilitating smoother acceptance by regulatory authorities [101].
- Flexibility in Validation: The validation process should adapt to the specific needs of the trial and evolving biomarker science, enabling the integration of innovative biomarkers [102].
- Focus on Trial-Specific Requirements: Streamlining the validation process by concentrating on the trial's specific needs as defined by the COU helps eliminate unnecessary steps and reduce associated costs.

4.3. Confirm validity using multi-faceted approaches

A comprehensive approach to validating PD biomarkers can improve their application in decision-making. Strategies include:

- Multimodal Techniques: Employ tissue-based, blood-based, and imaging biomarkers to provide a holistic view of drug effects. These methods help correlate clinical data with target modulation, enabling more informed decisions throughout the trial.
- PK/PD Modeling: Use pharmacokinetic/pharmacodynamic modeling frameworks to link biomarkers with trial outcomes. This may involve identifying patients with specific predictive biomarkers, ensuring active drug exposures, and measuring target engagement and pathway modulation.

4.4. Incorporate into adaptive trial designs

Real-time adjustments based on biomarker data can improve trial efficiency and increase success rates. Adaptive trials allow for interim analyses where accumulating data can be used to evaluate the performance of PD biomarkers in real time. For example, in a biomarker-guided combination therapy trial, small molecule biomarkers were used to monitor treatment response. As patients progressed through the trial, interval profiling of their biomarker signatures enabled researchers to identify which drug combinations were most effective for each individual. This approach validated the biomarkers by linking them directly to clinical outcomes. In addition, in first-in-human trials, exploratory PD biomarkers can be included to confirm the mechanism of action of candidate therapeutics. Techniques like Nanostring gene expression profiling, Luminex cytokine analysis, and flow cytometry were used to measure immune responses in blood samples. These biomarkers helped determine whether the drug was engaging its target, thereby validating their utility as PD markers [103].

4.5. Take the opportunity to explore and gain new insights

While robust, hypothesis-driven, quantitative biomarkers are primarily used to aid PK/PD modeling and inform dose

optimization decisions, clinical development proves a valuable opportunity to gain deeper insights into how treatment modulates disease pathways. By leveraging emerging technologies and exploring new hypotheses, researchers can probe changes in tumor microenvironment or shifts in the functional phenotypes of CAR-T cells in patients over time. These analyses can reveal important relationships between efficacy and potential resistance mechanisms, such as T-cell exhaustion [104].

Additionally, omics approaches – including proteomics, metabolomics, and transcriptomics – offer powerful tools to identify biomarkers that respond to drug treatment in a dose-dependent manner. Implementing such methods can uncover new layers in understanding biological responses to therapeutic interventions. Candidate biomarkers identified via omics approaches can be confirmed using targeted platforms amenable to FFP validation following guidelines mentioned above. For example, candidate PD biomarkers identified via whole transcriptome sequencing are often confirmed using validated qPCR-based methods to enable regulatory decision making.

By following the steps outlined above, PD biomarkers can be integrated effectively into clinical trials, while ensuring the assays are validated for their intended use and enhancing the drug development process. This integration supports better dose optimization, accelerates decision-making, and improves trial efficiency, ultimately contributing to safer and more effective treatments [105].

5. Early drug response monitoring

Target engagement analysis through PD biomarkers has evolved to focus on reliable, quantitative measurements that enable timely decision-making. Current approaches emphasize FFP validated assays under a pre-defined COU to provide reproducible and actionable data within timeframes relevant to development decisions [13]. Immunoassays measuring specific protein modifications, targeted LC-MS assays assessing protein degradation levels, and enzyme activity assays quantifying target inhibition provide definitive evidence of different kinds of target engagement within relatively short windows after dosing [5]. Similarly, cellular assays measuring functional responses offer reliable readouts of biological effect. Effective on-treatment monitoring requires assays with reproducible quantitation and clear response thresholds. On-treatment monitoring will allow establishment of temporal relationships between drug administration and biomarker changes to enable clinical program decision-making [106].

In drug development, PD biomarkers are instrumental in characterizing the onset, magnitude, and duration of pharmacological effects caused by the drug, guiding both dose and schedule optimization. Two recent examples of drugs that successfully leveraged PD biomarkers to inform Phase 3 dosing strategies are rusfertide and fazirsiran.

5.1. Case study 1

Rusfertide is a hepcidin mimetic peptide being studied as a potential new therapy for polycythemia vera (PV). Its MoA mimics hepcidin by inhibiting ferroportin on cells such as enterocytes, hepatocytes, and macrophages thereby constraining iron export into circulation and subsequently constraining erythrocytosis. While clinical benefits in PV patients are demonstrated by reduced phlebotomies or thrombotic events, circulating iron and transferrin-iron saturation (TSAT) serve as PD biomarkers to assess magnitude and kinetics of response in healthy volunteers. A single daily subcutaneous dose (1–80 mg) of an aqueous formulation resulted in dose-dependent reductions in serum iron and TSAT with a rapid onset, a nadir around 24 hours, a maximal effect with 20 mg, and a duration increasing with dose, supporting a weekly dosing regimen [107]. A Phase 2 study of rusfertide in PV patients used real-time monitoring of hematocrit as a PD biomarker to adjust the doses (10–120 mg) to maintain a hematocrit of less than 45% [108]. A lyophilized formulation of rusfertide was later developed, and serum iron and TSAT were again used as PD biomarkers to evaluate and compare the PK/PD relationship between the lyophilized and aqueous formulations [109]. Rusfertide is currently being evaluated in a randomized, double-blind, placebo-controlled Phase 3 study using hematocrit cutoff at 45% as a key secondary endpoint (NCT05210790).

5.2. Case study 2

PD biomarkers are especially valuable when drug effects are decoupled temporally from exposure, for example with RNAi therapeutics. RNAi therapeutics often display rapid declines in circulating drug exposure levels and delayed pharmacological effects due to the mechanism of first inhibiting gene transcription followed by a reduction in target protein levels. The kinetics of target protein reduction can vary widely based on factors such as transcript and protein stability; thus, having a noninvasive PD biomarker that can be measured repeatedly can be extremely helpful in understanding the exposure/response relationship and optimizing the dosing schedule.

Fazirsiran, a liver-targeted siRNA, is being investigated for alpha-1-antitrypsin deficiency-associated liver disease (AATDLD). In normal individuals, alpha-1 antitrypsin (AAT) is almost exclusively produced in the liver and is secreted into circulation where it travels to the lung and has a protective effect on lung elasticity. In patients with the homozygous PiZZ mutation, mutant Z-AAT protein polymerizes and accumulates in the liver, leading to fibrosis and liver disease. Fazirsiran causes degradation of AAT and Z-AAT mRNA, thus reducing protein synthesis in the liver and subsequently circulating protein levels.

Since liver fibrosis reduction – the clinical efficacy surrogate endpoint – requires years to manifest, PD biomarkers were critical for fazirsiran dosing optimization during early clinical trials. Following subcutaneous administration of a single dose (35, 100, 200 or 300 mg) in healthy volunteers, fazirsiran pharmacodynamics were evaluated by measuring serum AAT levels [110]. Repeated sampling revealed dose-dependent responses in serum AAT levels with a nadir at 6 weeks post-dose and a gradual rebound beginning at 12–16 weeks. These data informed Phase 2 designs that involved an injection

schedule of Day 0, Week 4, and then every 12 weeks for a year [111,112]. In Phase 2 studies, serum Z-AAT protein levels in patients harboring the PiZZ homozygous mutation were measured using an LC-MS/MS method that was sensitive and specific for a unique signature peptide containing the Z allele amino acid mutation. This PD biomarker analysis confirmed durable knockdown effects with quarterly dosing, forming the basis of an ongoing Phase 3 study (NCT05677971).

6. Recent advances, emerging trends, and alternative approaches for PD biomarkers

Recent technological advances in bioanalytical platforms and data analysis have accelerated the discovery of novel biomarkers. These innovations, along with our growing understanding of the human genome and proteome, have expedited the identification of novel biomarkers across therapeutic areas. This progress drives the demand for personalized treatments and targeted therapies, transforming disease diagnosis and management in clinical practice. Key trends are summarized in Table 2 and described below.

6.1. Artificial intelligence (AI)

The integration of multimodal data for biomarker discovery and prediction is a relatively new concept in precision medicine. AI techniques like Machine Learning (ML) and Deep Learning have been applied to various areas, including immuno-oncology and neurological disorders [135–137]. Biomarkers extracted from both retrospective and current clinical datasets hold promise not only for widespread use in personalized medicine but also for practical clinical applications. The appeal of AI-driven biomarker discovery strategies lies in their noninvasive nature compared to traditional sampling methods. Using AI-based pipelines to quantify chromatin biomarkers derived from published data on PBMCs from liquid biopsies, Challa et al. [113] demonstrated that the chromatin of cancer patients, across various tumor types, and those undergoing radiation therapy showed significant variations in their morphology and nuclear organization with remarkable accuracy, thereby facilitating assessment of treatment outcomes. Despite growing enthusiasm for using AI-driven biomarker research, several challenges continue to hinder its widespread adoption. Key obstacles include the integration of heterogeneous data types, limited availability of high-quality annotated datasets, and the opaque nature of many AI models, which reduces interpretability. Regulatory and ethical concerns such as data privacy, algorithmic bias, and unclear approval pathways further complicate clinical implementation. Additionally, AI models often struggle to generalize across diverse populations, and their integration into existing clinical workflows remains difficult. Infrastructure limitations and scalability issues also pose significant barriers to broader deployment [138,139]. As such, it is crucial to not only expand the accessibility of these methods but also to simplify their application, ensuring that personalized treatments can be optimized and cost-effective for a broader population.

6.2. Liquid biopsies

6.2.1. ctDNA

Circulating tumor DNA (ctDNA) has gained prominence as a leading biomarker in oncology for detecting, monitoring, and prognosing cancers (e.g., measurable/minimal residual disease (MRD)) [140–143]. These DNA fragments, typically no larger than 200 bp, are shed by tumor cells and exhibit mutations, methylation patterns, and other alterations [48,144]. Generally, the medium of choice for ctDNA extraction, liquid biopsies, which use blood, urine, or ascites fluid samples, offer significant advantages over traditional tissue biopsies, including easier accessibility and the ability to perform serial sampling to monitor disease progression. They also provide a more comprehensive disease heterogeneity profile. Recent technologies, such as CAPP-Seq and PhasED-Seq, enable ultrasensitive ctDNA detection, identifying tumor fractions with high specificity at levels as low as 1–2 parts per 10^6 –10^7 [145]. These platforms facilitate tumor genotyping without paired tissue biopsies, instead relying on peripheral blood mononuclear cells to capture germline gene signatures. The pivotal role of NGS technologies in advancing molecular oncology is underscored by the Association for Molecular Pathology and the College of American Pathologists' first guidelines for oncology panel validation, published in 2017 [21].

6.2.2. Exosomes

A promising choice for cancer diagnosis/prognosis are small membrane-bound vesicles (<200 nm in diameter) or exosomes that are shed by neoplastic cells [146,147]. These extracellular vesicles (EVs) carry a wide variety of biological material such as proteins, metabolites, and nucleic acid fragments (microRNAs, mRNAs, etc.), and may serve as rich sources of potential predictive and prognostic biomarkers in various cancer types [148,149]. In patients with colorectal cancer, Yoshioka et al. successfully demonstrated detection of CD147 and CD9 in plasma-derived EVs via a novel technology called ExoScreen [150]. Similarly, using ExoSearch method (utilizing a microfluic chip), Zhao et al. were able to measure exosomal-based ovarian cancer markers, CA-125, EpCAM and CD24 [151]. Interestingly, the combined use of blood-derived exosomal RNA and ctDNA greatly improved the sensitivity of EGFR mutation detection in NSCLC patients [152].

As exosomes shuttle a wide variety of intercellular cargo within the nervous system, they have been implicated in CNS development and some neurological diseases/disorders. For example, these extracellular vesicles (EVs) have been shown to play roles in inflammatory responses to glioblastomas and neuroinflammation such as that seen in Alzheimer's disease [153]. A positive correlation to disease progression and plasma exosome IL-6 has been demonstrated in ALS patients [153]. Exosomes also mediate complex pathological processes in Parkinson's disease, multiple sclerosis and other neurodegenerative disorders [153–155]. During CNS development, cell-cell interactions mediated by the EVs support cell proliferation and

Table 2. Recent advances, emerging trends and alternative approaches for PD biomarker analysis.

	Artificial intelligence – aided data analysis	Liquid biopsies: ctDNA and exosomes	High-dimensional flow cytometry and mass cytometry (CyTOF)	Imaging mass spectrometry (MSI) and spatial transcriptomics	Multi-omics	Multiplex IHC	Thermal stability shift assays
Sample matrix	Retrospective or recent data across multiple platforms and assays	Biofluid	Biofluid, cells, tissue	Cells	Biofluid, cells, tissue	Cells and tissue	Cells and tissue
Type of measurement	n/a	Qualitative, semi-quantitation	Qualitative, semi/quasi-quantitation or relative quantitation	Semi-quantitaton; can be used for relative quantitation with extra challenges	Qualitative/Semi-quantitation	Qualitative/Semi-quantitation	Qualitative/Semi-quantitation
Advantages	• *In silico* approach to analyze biomarkers from a wide variety of disease states using publicly available databases • Can integrate multiple and complex datasets to identify novel biomarkers with significantly improved accuracy • Enhanced treatment outcome predictions via superior diagnostic precision • Capable of significantly improving disease prognosis predictions • Relatively faster turn-around time to enable Go/No-Go decisions • Can be personalized or tailor-made for specific disease states or populations • Can greatly optimize clinical trial design	ctDNA: • High sensitivity and specificity. Can detect 1 part per million copies or less • Facilitates serial sampling from a wide range of body fluids enabling robust tracking of treatment response • Some of the emerging platforms do not require paired biopsies for germline genotyping, making sample analysis easier and more patient friendly • Comprehensive profiling of disease heterogeneity • Ability to predict disease relapse much earlier than other orthogonal methods (e.g., flow cytometry, PCR, NGS); high abundance, easy to detect Exosomes: • Rich source of a variety of disease-specific biomarkers (protein, DNA and RNA fragments) • Relatively stable as they are vesicular with higher abundance as they are secreted by many types of cells	High-dimensional flow cytometry: • Significantly deeper phenotypic and functional characterization potential at the single-cell level providing more information on the cell populations under study • Increased number of cellular markers analyzed in a single sample potentially decreases the sample volume and reagent requirements, sample processing time • Expanded panel capabilities enables assay use across different studies Mass cytometry: • High dimensional mass analysis with single-cell resolution to provide detailed landscape of cell heterogeneity • Multiplexing capability with minimal signal overlap by using rare earth metal isotopes as tags. • Cell surface protein quantitation	MSI: • Label-free detection of broad molecular coverage, including metabolites, lipids, peptides, small proteins and therapeutics • Preserve tissue architecture to maintain spatial context for site-specific pharmacodynamic interrogation Spatial transcriptomics: • Allows for mapping and studying distributions of gene expressions (RNA transcripts) in a spatial context • Reveals tissue architecture, cell structures and cell-cell interactions • Amenable to AI due to the high-content nature of the data	• Comprehensive molecular profiling enables the discovery of multiparametric biomarker signatures, both for efficacy and safety • Data can be used to inform MoA • Amenable to AI due to the high-content nature of the data • In late development, can be used to inform combination therapies	• Detect and analyze multiple biomarkers simultaneously • Visual confirmation and co-localization of biomarker expression on tissues. Tissue sections • Single-cell resolution with 2D spatial information	• Based on physical-chemical properties, independent of biological responses. • Easy laboratory set-up • Flexible in detection methods and readout format • High-throughput
Challenges and limitations	• Relies heavily on accessibility to large datasets of high quality • Requires extensive and complex programming skills for data mining • Nascent technology with limited clinical validation	ctDNA: • Not a one-size-fits-all; analysis pipeline algorithms and library prep are target-specific • Niche technologies leads to greatly reduced global footprint; accessibility to the technologies may be limited • May not be cost-effective • Turn-around time longer than most traditional biomarker assays	High-dimensional flow cytometry: • Increased complexity in panel design, assay development, and validation can extend timelines and cost • Volume of data acquired is very large, requiring advanced analysis approaches, extended analysis time and standardization • Highly specialized expertise required	MSI: • Dependent on availability of antibodies; in situ digestion included for proteins • Can only analyze analyte up to 25kDa, though newer advances allow higher molecular weight cutoff with lowered resolution and sensitivity • Complex sample preparation workflow • Large data complexity and size require adequate data storage and management systems	• Complexity of data necessitates specialized expertise and advanced data infrastructure • Requires large investment in resources	• Availability of tissue material: quality and quantity • Limited serial sampling • Availability of high-quality critical reagents such as antibodies • Lower throughput • Inter-laboratory reproducibility and analysis standardization	• Not applicable for every protein – only demonstrated with soluble protein binding with low molecular weight compounds • License purchase from vendor is required for use

(*Continued*)

Table 2. (Continued).

	Artificial intelligence – aided data analysis	Liquid biopsies: ctDNA and exosomes	High-dimensional flow cytometry and mass cytometry (CyTOF)	Imaging mass spectrometry (MSI) and spatial transcriptomics	Multi-omics	Multiplex IHC	Thermal stability shift assays
		Exosomes: • Time-intensive and labor-intensive isolation process • The isolation process is challenging to automate and scale up due to their size and heterogenity • Kit based protocols may decrease isolated exosome purity (contaminations from other membrane-bound vesicles) • Low reproducibility • Difficult to perform quantitation due to lack of normalization factors • Difficult to discern the tissue origin of the exosome to yield significant biological meanings • Lack of vendors for clinical trials • May require large volume of starting material (matrices) pending on exosome isolation yield and analyte concentration	Mass cytometry: • Slower acquisition speed might limit the number of samples being analyzed • High cost of specialized instrument; requires dedicated SME for both instrument operation and data analysis • Antibody availability with metal conjugation capability	Spatial transcriptomics: • Lower transcript coverage and depth, poor resolution with low gene expression levels • High quality sample preservation and preparation required • Not cost-effective			
Area/s of application	Biomarker and target identification, disease prognosis, personalized medicine, patient stratification	Measurable/Minimal Residual Disease (Oncology)	Biomarker discovery, target engagement, pathway modulation, spatial biomarker identification, mechanistic interrogation, disease biology investigation, phenotypic and functional profiling	MSI: Spatial biomarker identification, mechanistic interrogation, disease biology investigation, phenotypic and functional profiling Spatial Transcriptomics: Oncology, Developmental biology, Neuroscience	Biomarker and target identification, mechanistic interrogation	Biomarker and target identification	Biomarker and target identification
Representative applications	[113,114]	[115,116]	[117–122]	[123–125]	[126–130]	[131,132]	[133,134]

neurogenesis, differentiation and formation of synapses [156]. Thus, exosomes exist to serve a dual purpose as markers of both disease and development.

6.3. High-dimensional flow cytometry and mass cytometry

Expanded capabilities with high parameter flow cytometry instrumentation and reagent availability allow for multidimensional analysis facilitating deeper and more detailed characterization of cell populations and offers additional opportunities to use PD biomarkers to advance drug development [157,158]. Incorporating AI/ML approaches can facilitate the analysis of these large datasets (see Table 8 in Czechowska et al., 2025 for examples of available tools) [159]. Combining flow cytometry and mass spectrometry (e.g., CyTOF) quantifies labeled targets on or within cells, providing immunophenotype and functional characterization at single-cell resolution [158,160]. Recent studies using CyTOF have measured the number of target-specific antibodies bound per circulating cell in multiplexed formats, determining membrane target expression levels [161]. Imaging mass cytometry can support >40 biomarkers in single sections and is commercially available [162,163].

6.4 Imaging mass spectrometry

Similarly, mass spectrometry imaging (MSI) (e.g., TIMS-TOF, MALDI-TOF) generates spatial distribution data for small molecules, lipids, proteins, and metabolites in tissue samples, offering insights into target engagement and pharmacodynamic responses [164,165]. Though currently limited to exploratory preclinical studies due to analyte and sample constraints, MSI is invaluable for understanding disease biology and drug effects. A new review by Korber et al. highlights recent improvements and increased capabilities for imaging mass spectrometry [166]. As datasets expand, AI/ML and automation are expected to enhance data analysis, uncovering deeper insights into biological phenomena.

6.5. Higher sensitivity immunoassays

Newer technologies for immunoassays provide 1) ultra-high sensitivity down to 10 femtograms/mL in the case of Simoa® (Quanterix), and 1 picogram/mL in the Ella™ (ProteinSimple) platform; 2) multiplexing capabilities as with Luminex xMAP [167], and S-Plex (Meso Scale Discovery; MSD) or even higher multiplexing capabilities that can be obtained with Olink's proximity extension assay (PEA) technology [139,140]; and 3) the possibility of wider dynamic ranges that can cover up to 4–5 log units, e. g., Simoa® (Quanterix), MSD (Meso Scale Discovery) and Ella™ (ProteinSimple). Additionally, automation features in platforms like Gyrolab® (Gyros Protein Technologies) and Ella™ streamline and accelerate sample analysis. Innovations in hybrid technologies are advancing both sensitivity and multiplexing potential. For instance, the NULISA™ platform (Alamar Biosciences), which combines antibody and qPCR (singleplex) or NGS (multiplex) technology, offers an extended dynamic range for biomarker quantitation while maintaining ultra-high sensitivity [168].

6.6. Multi-omics

Multi-omics, including genomics, transcriptomics, proteomics, metabolomics, and lipidomics, individually or in combination, serve as powerful technologies to unravel biological complexity and understand drug impact on modulating disease pathways at a molecular level [126]. In early R&D, high-throughput advancements enable profiling of lead series to assess on-target versus off-target effects, establishing valuable PD and "safety signals" for ongoing preclinical and clinical program monitoring [127]. When combined with AI, multi-omics has the potential to inform therapeutic index optimization at an early stage, thereby increasing the likelihood of success [128]. Furthermore, integrating multiple omics approaches enhances confidence in the observed molecular changes, and offers flexibility in targeted biomarker assay development (e.g., selecting between transcripts and proteins as readout). In development, multi-omics can also play a significant role in clinical trials. Precision medicine in particular benefits from this approach, enabling the tracking of single or combined response biomarkers to assess patient-to-patient variability in treatment outcomes [129,130].

6.7. Spatial transcriptomics

Spatial transcriptomics has transformed tissue morphology analysis, moving from 2D visualization to more complex 3D views [169,170]. This technology allows for detailed exploration of gene expression, spatial gene location, tissue composition, and intercellular interactions. Techniques such as single-cell RNA sequencing, high-definition spatial transcriptomics (in-situ capture), ISH, and FISH are revolutionizing understanding in diverse disease areas, including neurodegeneration, oncology, and cardiovascular research [171].

6.8. Multiplex IHC

Multiplex IHC provides the ability to analyze multiple biomarkers from limited tissue sections – a critical advantage when specimen availability is scarce [162,172,173]. While its capabilities are constrained by the number of chromogens usable simultaneously, immunofluorescence enhances this technology with fluorescence signal dynamics, enabling semi-quantitative analysis. InSitu Plex® technology (Ultivue) represents a significant advancement, using DNA-barcoded antibodies for single-amplification followed by fluorescent-tagged probe binding for imaging. CODEX (Co-detection by indexing) extends these capabilities, allowing visualization of up to 60 biomarkers in single tissue sections at single-cell resolution, as demonstrated in colorectal cancer specimens [174]. Multiplexed Ion Beam Imaging (IONpath, Inc.) further pushes this field, detecting > 40 cellular markers with spatial data at subcellular resolution, as validated in diverse tumor types [175].

6.9. Thermal stability shift assays

Thermal stability shift assays exploit changes in protein stability upon ligand binding [176]. Originally developed for high-throughput drug screening [177,178], this method has evolved

for use in complex biological matrices, facilitating target engagement studies. CETSA® (CEllular Thermal Stability Assay), licensed through Pelago Bioscience, offers label-free target engagement measurements *in vivo*, coupled with various detection methods (e.g., MS, AlphaLISA, WB). Recent advancements in CETSA® coupled with MSD highlight its applicability in preclinical and clinical settings [179], showcasing its potential for clinical trial adoption.

7. Conclusion

PD biomarkers have emerged as transformative tools in drug development, providing critical insights into drug mechanisms, patient responses, and therapeutic efficacy and safety. They can play a pivotal role in regulatory approvals, particularly in rare disease indications, and are indispensable in assessing treatment impact, optimizing dosing strategies, and guiding decisions on program progression or termination. The successful implementation of PD biomarkers hinges on robust bioanalytical methods and rigorous assay validation to ensure the generation of reliable and actionable data.

8. Future perspective

Technological advancements have vastly expanded opportunities for the innovative application of PD biomarkers in clinical development. These biomarkers now serve as research tools for identifying novel candidates and generating insights that can further enhance drug characterization. Cutting-edge measurement techniques such as NGS, multiplex IHC, spatial imaging, and transcriptomics have improved biomarker detection and analysis, offering unmatched contextual information. Computational advances like machine learning and artificial intelligence are reshaping data interpretation, enabling more precise and predictive analyses of intricate biological systems. These advances will reduce clinical program duration, improve quality of data, and provide researchers with a deeper understanding of biological pathways and diseases mechanisms.

Furthermore, the growing integration of real-time monitoring through wearable biosensor technologies enables the collection of digital biomarker data that complements traditional assay data [180]. Digital biomarkers have the potential to revolutionize drug development as they can allow drug developers to assess functionality or physiological responses that were previously immeasurable [181]. They also allow for effortless collection of thousands of previously unobtainable data points while minimizing the impact of this substantial data collection on patients [182]. As these technologies evolve, so must the regulatory frameworks necessary to accommodate their use. Cross-sector collaboration is essential to ensure the successful development of standards and implementation of any new technology into clinical practice.

The proactive integration of a well-designed PD biomarker strategy facilitates the effective translation of research findings into clinical settings which enhances clinical trial efficiency and reduces development costs, thereby expediting the introduction of innovative and effective therapies for patients.

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