



## REVIEW ARTICLE

## Analytical Validation of Quantitative Pharmacodynamic Methods used in Clinical Cancer Studies

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### Abstract

Quantitative pharmacodynamic (PD) methods are used to assess the effect of a drug on its target. The use of these methods in clinical cancer studies has increased enormously with the advent of targeted therapies in the past years. To ensure that quantitative PD methods meet their expectations, methods need to be validated according to internationally recognized standards for amongst others specificity, accuracy, sensitivity, precision and stability. We show that 87% of our selected clinical cancer studies, published in the last 7 years, used quantitative PD methods that were on average only validated for half of the 10 main validation parameters. Here, we offer our view on the quality of, and the challenges encountered in validation of quantitative PD methods. Finally, we provide future directions and considerations for improvement of the method validation quality.

### Keywords

Quantitative, Pharmacodynamic, Biomarker, Validation, Guidelines, Clinical, Cancer

### Introduction

The need for pharmacodynamic (PD) biomarker methods continues to grow with the explosive increase of targeted agents in clinical cancer therapy. PD biomarkers are endogenous biological indicators that can be measured objectively and provide insight about the target engagement, and proof of concept i.e. the extent of the biological effect of a drug. Poor target validation or insufficient target engagement is the main reason for failure of 42% of clinical phase I and 63% of phase II clinical cancer trials [1]. Early recognition of these failures is important, which can be accomplished by using PD biomarkers to provide proof of mechanism

for the drug-target engagement. PD biomarkers may include molecular, histologic, radiographic, or physiologic characteristics. Most PD biomarkers are, however, molecular biomarkers, which encompass among others protein modifications, enzyme activities, receptor expression and occupancies, and cell counts. In early clinical trials, PD biomarkers can provide useful information for patient management, e.g., whether to continue treatment or to adjust dose (schedule) [2]. They can also help to guide drug dose individualization in targeted/personalized cancer therapy with the aim of treating cancer more effectively and with less toxicity [3]. An illustrative example of the potential usefulness of PD evaluations is the monitoring of B-lymphocyte suppression as PD biomarker in clinical trials to find doses of anti-CD20 monoclonal antibodies required to maximally reduce this cell population. The reduction of the amount of B-cells is presumed to underlie the clinical benefits of these drugs in treating cancer, and has become an established indicator for treatment success [4]. Another example is the monitoring of the inhibition of PARP (target) enzyme activity in white blood cells as PD biomarker for the target engagement of the anti-cancer drug Olaparib [5].

Quantitative PD biomarker methods are used to determine biomarker activity or concentration in different biological matrices such as blood, bile, serum, plasma, urine, tumor cells, and tissue such as skin. For this purpose, these methods need calibration standards prepared in biomarker-free surrogate matrices that are fully defined and representative for the measured biomarkers and their biological matrices. Full analytical



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validation of these methods is essential to assure accurate and specific biomarker quantification to prevent interference from other compounds in these matrices. Often biomarkers can fail not because of the underlying science, but because of poor choice of methods and lack of analytical validation [2]. The development and validation of quantitative biomarker methods is, however, more complicated than that of bioanalytical drug methods used for pharmacokinetics due to the following complications. Firstly, sometimes biomarkers need to be detected in more complex biological matrices such as tumor tissues. Secondly, the stability of biomarkers and other endogenous compounds in the biological matrix is often poor. Thirdly, biomarkers are endogenous substances, thus obtaining biomarker-free surrogate matrices for validation of method specificity, and preparation of the calibration curve may be more difficult. Lastly, the biomarker molecule is often not available to act as a certified calibration standard [6].

The last decade has seen significant progress in harmonisation of acceptance criteria, definitions, and guidelines for bioanalytical method validation (BMV) of chromatography and ligand binding methods [7]. For this purpose, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recently released BMV Guidance M10 [8]. Fundamentally, ICH-M10 validation involves demonstration of bioanalytical method specificity, accuracy (mean % deviation from nominal concentration), linearity, dilution integrity, sensitivity, limit of quantification, precision (% coefficient of variation (CV), and stability for drugs (Table 1). However, international harmonization of guidelines for biomarker method validation is lacking. In their BMV 2018 guideline, the Food and Drug Administration (FDA) recommends to “conduct a full validation of any

new bioanalytical method for the analysis of a new drug entity, its metabolite(s), or biomarkers.” [9]. The validation of biomarkers thus needs to be consistent with the validation principles applied to bioanalytical drug methods validation [9]. This guideline further states that exploratory quantitative biomarker methods, that are not used to support regulatory decision making, may not require such stringent validation. Exploratory biomarker methods are for instance used to better understand the mechanism of action of drugs, which may ultimately lead to improved future cancer treatments. However, in light of their importance, and to make sure that these methods live up to their expectations, we would argue that also exploratory quantitative biomarker methods, used in clinical trials, need to be fully validated.

Herein, we present our viewpoint on the degree of compliance with the FDA BMV 2018 guideline of quantitative PD biomarker methods used in our selection of clinical cancer trials. Challenges encountered in the process of PD biomarker method development and validation are discussed. The implications of poorly validated methods used for assessment of drug effects are described. In the final section, we offer some considerations and suggestions that may help to improve the quality of PD biomarker methods and consequently their usefulness in clinical cancer trials.

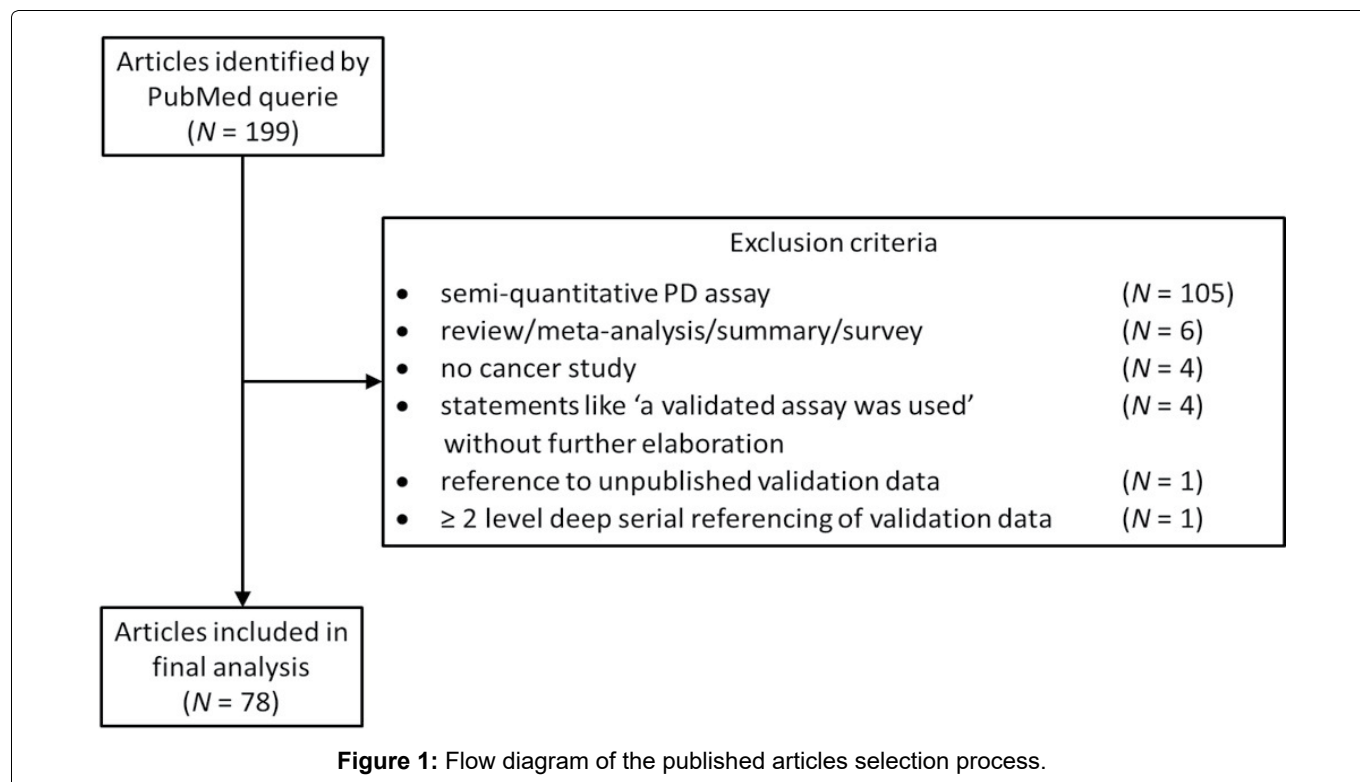
## Analytical Methods Validation

For the assessment of the status of analytical validation of quantitative PD methods used in clinical cancer trials, a focused PubMed literature query was performed on the 8<sup>th</sup> of December 2020, including the keywords “2013/04/12” [PDAT] : “2020/12/08” [PDAT] AND “humans” [MeSH Terms] AND (“Clinical Trials, Phase I as Topic” [Mesh] OR “Clinical Trials, Phase II as Topic” [Mesh] OR “Clinical Trials, Phase III as Topic”

**Table 1:** Validation parameters.

Validation Parameters		Definition and Criteria
I	Specificity	Ability to detect and differentiate biomarker from other (related) substances
II	matrix effects	Interfering sample matrix component(s) may not affect accuracy
III	Accuracy	Measured and nominal biomarker concentration differ $\leq 15\%$
IV	Dilution linearity*	Extra dilution of quality controls exceeding ULOQ is accurate and precise
V	Dilution integrity#	Normal dilution of samples is accurate and precise
VI	Sensitivity/LOD	Lowest biomarker concentration that is significantly above background level
VII	LLOQ	Lowest concentration that can be measured with accuracy and precision $\leq 20\%$
VIII	Precision	Variation in concentration between repeated measurements $\leq 15\%$
IX	Quality controls	Prepared in matrix independently from standards; run together with samples to assure validity; should be within 20% of their nominal concentration
X	Storage stability	Effect storage conditions on biomarker concentration $\leq 15\%$

Definition and main criteria of the biomarker validation parameters from the Food and Drug Administration (FDA) Bioanalytical Method Validation (BMV) guideline 2018 [5]. \*Because of the narrow range of the calibration standard curve, it is necessary to demonstrate with quality control samples that the analyte of interest, when present in concentrations exceeding the range of quantification (above ULOQ), can be accurately measured by the assay after dilution in blank matrix to bring the biomarker concentrations into the validated range for analysis. An additional reason for conducting dilution linearity experiments is to detect a possible prozone or “hook effect” for ligand binding assays. #The effect of dilutions used for samples with normal biomarker concentrations on accuracy and precision. LOD = limit of detection; ULOQ and LLOQ = upper and lower limit of quantification.



[Mesh] OR "Clinical Trials, Phase IV as Topic" [Mesh] OR phase I [tiab] OR phase 1 [tiab] OR phase II [tiab] OR phase 2 [tiab] OR phase III [tiab] OR phase 3 [tiab] OR phase IV [tiab] OR phase 4 [tiab] OR "Clinical Trials, Phase I as Topic" [Mesh] OR "Clinical Trials, Phase II as Topic" [Mesh] OR "Clinical Trials, Phase III as Topic" [Mesh] OR "Clinical Trials, Phase IV as Topic" [Mesh]) AND (pharmacodynamic\* [tiab] AND method AND methods AND cancer NOT modeling [tiab] NOT review [tiab]). The query returned 199 articles. After review, 4 articles were excluded for failure to provide details about used validation criteria and guidelines. Another article was excluded for referencing to validation data in a submitted, but never published, other article from the authors. Yet another article was excluded for serial referencing. After applying additional exclusion criteria, 78 (39%) of articles were found suitable for our evaluation using quantitative PD methods in human clinical cancer studies (Figure 1) [10-87]. From each of these articles one quantitative PD method was evaluated and qualitatively scored for compliance with the 10 main validation criteria defined by the FDA BMV 2018 guideline (Table 1 and Table 2).

We found that, 10 PD biomarker methods (13%) were fully validated in accordance with all 10 main FDA BMV 2018 validation parameters. However, 53 PD methods (68%) were validated for just on average 5 parameters. Total absence of PD method validation was found for 17 (22%) of published clinical studies. Compared to chromatographic and ligand binding methods, flow cytometry (FC) methods were relatively less well validated. FC methods were validated for on average just 2 parameters and 50% lacked any validation. In order to effectively utilize biomarkers to

draw meaningful conclusions, it is imperative that the measured concentration or activity of the biomarker in study samples is as close as possible to the actual value at the time of sample collection. The FDA guideline states that investigators should always evaluate their PD methods for stability themselves, referencing the limited sample stability data published in literature is not considered sufficient [9]. For this purpose, stability needs to be assessed for all stocks and reagents used, and for biomarkers during all different parts of the experimental procedure: 1) From sample collection in the clinic to the start of the sample work-up in the lab. 2) On the bench-top or in the autosampler after sample work-up. 3) Long term storage. 4) Freeze-thaw stability. Stability during the sample work-up procedure is by definition not part of the validation, but should be assessed during method development. Nevertheless, only 26 PD methods (33%) were evaluated for reagent and stock solution stability, and biomarker stability during one or more parts of the experimental procedure.

### Challenges and Considerations

Commercial immuno-assay kits were used in 33 (77%) of PD methods. These kits are usually developed for general use and therefore may need some level of optimisation, such as adding more calibrators and/or replacing the kit controls in a buffer with controls that mimic the sample matrices. This may prevent accuracy problems from a lack of parallelism between standard curves prepared in buffer and matrix. Many reagents are "research purpose only" (RUO) with considerable batch to batch variation. The use of good manufacturing practice (GMP) reagents or pooled RUO batches should be preferred if possible. A common complaint from

Table 2: Analytical methods validation.

Biomarkers (alphabetically)	Assay	Origin	Validation parameters										Drug	Phase	Year	Ref #	
			I	II	III	IV	V	VI	VII	VIII	IX	X					
ADA	ELISA	LDA	+	+	+	+	+	+	+	+	+/+	+	+	Blinatumomab	1	2018	34
ADA	LBA	Comm.	+	+	+	+	+	-	+	+/+	+	+	+	Regorafenib	1	2016	13
ADA	ELISA	LDA	-	-	-	-	-	+	-	-/-	-	-	-	TAS-266	1	2015	75
ADA	ELISA	LDA	-	-	-	-	-	+	-	-/-	-	-	-	Angiocal	1	2013	67
Ang2	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	+	Docetaxel	2	2014	52
B/T-cells	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	Birinapant	2	2016	86
BTK	LBA	LDA	+	-	+	+	-	+	+	+/+	-	+	+	Tirabrutinib	1	2018	26
BTK	ELISA	LDA	+	-	-	-	-	+	-	-/-	-	-	-	Acalabrutinib	1	2018	25
ccK18	ELISA	Comm.	+	+	+	+	+	+	+	-	+/+	+	+	SOR-C13	1	2017	51
CD137	xMAP ELISA	Comm.	+	-	+	+	+	+	+	+	+/+	+	+	Utomilumab	1	2018	56
CD26	FC	LDA	+	-	-	-	-	-	-	-	-/-	-	-	YS-110	1	2017	58
CD3/14/20 cells	FC	LDA	-	-	-	+	-	-	-	-	-/-	-	-	CC-223	1	2015	45
CEC	FC	LDA	+	-	+	-	-	-	-	-	+/+	-	-	Pazopanib	1	2014	36
CgA	RIA	Comm.	-	-	-	-	-	-	+	+/+	-	-	-	Lanreotide	3	2016	31
Collagen IV	ELISA	Comm.	+	-	-	+	-	+	-	-/-	-	-	-	Cediranib	1	2015	35
CRP	LBA	n.d.	-	-	-	-	-	-	+	-/-	-	-	-	Siltuximab	1	2013	21
CTC	FC	Comm.	+	-	+	+	+	+	+	+/+	+	+	+	ASP-9521	1/2	2014	85
CTC	FC	Comm.	+	-	+	+	+	+	+	+/+	+	+	+	ARN-509	1	2013	44
CTC	FC	LDA	-	-	-	-	-	+	-	-/-	-	-	-	Alisertib	1/2	2017	79
CTC	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	TRC105	2	2016	61
mCTC	FC	LDA	+	+	+	+	+	+	+	+/+	+	+	+	5-F-2'-dC	1	2020	24
CTX1	ELISA	Comm.	+	-	-	+	-	+	+	+/+	+	-	-	GLPG-0187	1	2016	17
Cytokines	xMAP ELISA	Comm.	+	-	+	+	+	+	+	+/+	+	+	+	TMX-101	1	2015	10
dFdC-TP	LC-MS/MS	LDA	+	-	+	+	+	+	+	+/+	+	+	+	Gemcitabine	1/2	2015	38
DNA	LC-MS/MS	LDA	+	+	+	+	+	+	+	+/+	+	+	+	LY2603618	2	2017	62
EGF	LBA	LDA	-	-	-	-	-	-	-	-/-	-	-	-	Sorafenib	1	2015	37
EPO	LBA	Comm.	+	+	+	+	-	+	-	+/+	+	-	-	PT2385	1	2018	66
FGF	ELISA	Comm.	+	-	-	+	-	-	+	+/+	-	-	-	S49076	1	2017	70
FGFR	ELISA	LDA	-	-	-	-	-	-	-	-/-	-	-	-	JNJ-42756493	1	2015	71
G-CSF	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	-	-	Lenvatinib	1	2013	43
GSH-GSSH	Spectrometry	Comm.	-	-	-	-	-	+	-	-/-	-	-	-	IACS-010759	1	2020	30
γ-H2AX	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	MK-8776	1	2015	42
HSP70	ELISA	n.d.	-	-	-	-	-	-	-	-/-	-	-	-	NVP-AUY922	2	2014	77
γ-IF	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	Cixutumumab	2	2014	12
IGF1	ELISA	Comm.	+	-	+	+	-	+	-	+/+	+	-	-	Polyphenon E	2	2017	39
IGF1	ELISA	Comm.	+	-	+	+	-	-	+	+/+	+	-	-	MEDI-573	1	2015	54
IGF1R	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	BIIB-022	1	2014	72
IGFBP2	ELISA	Comm.	+	+	+	+	+	+	-	+/+	+	-	-	CNF-1010	1	2013	65
II-2	ELISA	n.d.	-	-	-	-	-	-	-	-/-	-	-	-	RO4929097	2	2015	29
II2-10	ELISA	Comm.	-	-	-	-	-	-	-	-/-	-	-	-	Ponatinib	2	2019	74
II-8	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	+	Sunitinib	3	2013	87
LFA102	ELISA	LDA	-	-	-	+	-	-	-	-/-	-	-	-	LFA-102	1	2016	83
lymphocytes	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	Dinaciclib	1	2013	33
monocytes	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	-	Emactuzumab	1	2019	19
p-AKT	ELISA	Comm.	-	+	+	+	+	+	-	+/+	+	-	-	PX-866	2	2019	82
p-AKT	xMAP ELISA	Comm.	+	-	+	+	-	+	-	+/+	-	-	-	Pictilisib	1	2016	41
p-AKT	xMAP ELISA	Comm.	+	-	+	+	+	+	+	+/+	+	+	+	Pictilisib	1	2015	59

PAR	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	Olaparib	1	2018	16
PAR	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	ABT-888	1	2017	57
p-BAD	ELISA	Comm.	+	-	-	+	-	+	-	+/-	-	-	AZD1208	1	2018	46
p-CREB	FC	LDA	+	-	-	-	-	-	-	+/-	-	-	AB-928	1	2018	80
PD1	ELISA	LDA	+	+	+	+	+	+	+	+/+	+	+	SHR-1210	1	2018	18
PDGF	LBA	Comm.	+	-	-	+	+	-	+	+/+	+	-	Motesanib	2	2015	48
PDGF	xMAP ELISA	Comm.	+	-	+	+	+	+	+	+/+	+	+	MEDI-575	1	2014	40
PDGF	ELISA	Comm.	+	+	+	+	+	+	-	+/+	+	-	Pazopanib	1	2013	47
PD-L1	FC	LDA	+	-	-	-	-	-	-	-/-	-	-	Velumab	1a	2017	53
p-ERK	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	TAK-733	1	2016	73
p-FAK	ELISA	Comm.	+	-	+	+	+	+	-	+/+	+	-	BI 853520	1	2019	60
PI3K	FC	LDA	-	-	-	-	-	-	-	-/-	-	-	Temsirolimus	1	2017	78
p-RAD50	MRM-MS	LDA	+	-	+	+	+	+	+	+/+	+	+	AZD0156	1	2018	14
PRAS40	ELISA	Comm.	+	-	+	+	+	+	-	+/+	+	-	Azacitidine	1	2017	32
p-SCR	ELISA	Comm.	+	-	-	-	-	-	-	-/-	-	-	Dasatinib	2	2019	23
Rhodamine	Spectrometry	LDA	+	-	+	-	-	-	-	+/+	-	-	Tariquidar	1	2015	68
RON8	ELISA	Comm.	+	-	-	+	-	-	-	-/-	-	-	Narnatumab	1	2017	55
Serum IgG	xMAP ELISA	Comm.	+	-	+	+	+	+	+	+/+	+	+	Sipuleucel-T	3	2015	15
serum proteins	xMAP ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	Cediranib	3	2014	84
s-VEGF	ELISA	Comm.	+	-	+	+	-	+	+	+/+	+	-	Bevacizumab	2	2014	81
s-VEGF1/2	ELISA	n.d.	-	-	-	-	-	-	-	-/-	-	-	Lenvatinib	1	2014	69
s-VEGF1/2	ELISA	Comm.	+	+	+	+	+	+	+	+/+	+	+	Ramucirumab	3	2020	22
s-VEGFR2	ELISA	n.d.	-	-	-	-	-	-	-	-/-	-	-	XL-647	1	2018	49
Testosterone	HPLC	LDA	+	-	+	+	+	+	+	+/+	+	+	Abiraterone	2	2018	64
Testosterone	LC-MS/MS	LDA	+	+	+	+	+	+	+	+/+	+	+	Relugolix	1	2015	11
Testosterone	LC-MS/MS	LDA	-	-	-	-	-	-	+	-/-	-	-	Orteronel	1	2015	27
TSA	radio-assay	LDA	+	+	+	+	+	+	+	+/+	+	+	Capecitabine	1	2020	63
VEGF	ELISA	Comm.	+	-	+	+	-	+	+	+/+	+	+	Cediranib	1	2015	76
VEGF	ELISA	Comm.	+	-	+	+	-	-	+	+/+	+	-	TRC105	1	2015	20
VEGF-A	LBA	LDA	-	-	-	-	-	-	-	-/-	-	-	Ramucirumab	1	2015	50
virus infectivity	Microscopy	LDA	-	-	-	-	-	-	-	-/-	+	-	Enadenotucirev	1/2a	2019	28

Qualitative validation scores for PD assays from 78 phase 1-3 clinical cancer studies. (+) validation in accordance with the Food and Drug Administration bioanalytical method validation guidelines from 2018 [5]; (-) not validated; Ref.# = article reference number; validation parameters: I = specificity, II = matrix effects, III = accuracy, IV = dilution linearity, V = dilution integrity, VI = sensitivity/limit of detection, VII = lower limit of quantification, VIII = within- and between day precision, IX = quality controls, X = sample storage stability; ADA = anti-drug antibodies; Ang-2 = Angiopoietin-2; BTK = Bruton's Tyrosine Kinase; cck18 = cleaved cytokeratin 18; CD = cluster of differentiation; CgA = chromogranin A; CRP = C-reactive protein; mCTC = mesenchymal circulating tumor cells; CEC = circulating endothelial cells; CTX-1 = C-terminal telopeptide of type I collagen; Comm. = commercial assay; EGF = epithelial growth factor; ELISA = enzyme linked immunosorbent sandwich assay; EPO = Erythropoietin; dFdC-TP = gemcitabine triphosphate; FC = flow cytometry; FGFR = fibroblast growth factor receptor; G-CSF = granulocyte colony stimulating factor; GSH-GSSG = glutathione reduced and oxidized;  $\gamma$ -H2AX = phosphorylated variant histone 2A; HPLC = high performance liquid chromatography; HSP70 = heat shock protein 70;  $\gamma$ -IF = gamma interferon; IGF1R = insulin growth factor receptor; IL = interleukin; LFA = Prolactin Receptor Antagonist antibody; LBA = ligand binding assay; LDA = lab developed assay; LC-MS/MS = liquid chromatography tandem mass spectrometry; MRM-MS = multiple reaction monitoring mass spectrometry; n.d. = not disclosed p-AKT = phosphorylated serine/threonine-protein kinase; PAR = poly-adenosine diphosphate ribose; p-BAD = phosphorylated B-cell lymphoma antagonist of cell death; p-CREB = cAMP Response Element Binding protein; PD1 = programmed death; PDGF = platelet derived growth factor; PD-L1 = programmed death ligand; p-ERK = extracellular-signal-regulated kinase; p-FAK = Focal adhesion kinase; PI3K = phosphatidylinositol 3-kinase; p-RAD50 = double strand break repair protein; PRAS40 = proline-rich serine/threonine-protein kinase substrate; p-SCR = serum creatinine; RON8 = Macrophage-stimulating 1-receptor; VEGFR = vascular endothelial growth factor receptor; TSA = thymidylate synthase activity; RIA = radioimmunoassay; xMAP = multi-analyte profiling.

scientists is that suppliers do not provide the data required to evaluate a given antibody's specificity or its lot-to-lot variability [88]. Companies might ship a batch of antibodies with characterization information derived

from a previous batch [88]. And any validation data that are given are often derived under ideal conditions that do not reflect typical experiments [89]. Investigators should be aware that certain suppliers are releasing with

a fast pace antibodies and ELISA kits of questionable quality [88]. The largest vendors often buy antibodies from smaller suppliers, relabel them and offer them for sale [88]. Such products can lead to unfounded conclusions, waste of many months of research and publications that subsequently need to be retracted. In one illustrative example, the investigators spent 2 years and \$500,000 because the commercial kit they were using did not recognize Zona Pellucida-like domains protein 1 but Cancer Antigen 125 [90]. Investigators should realise that validation of specificity is the basis of every reliable method. Ideally, researchers would refuse to buy antibodies and immune-assays without extensive validation and quality control data, or would perform the validation themselves. Antibodies should be tested for off-target binding using positive and negative controls e.g. control experiments that involve engineering cell lines to both express and stop expressing the protein of interest. We recommend additional in-house validation before using commercial antibodies and kits, also when obtained from suppliers with a proven track-record, and subsequent entry of the in-house validation data in centralized antibody validation registries such as CiteAb ([www.citeab.com](http://www.citeab.com)) [91]. CiteAb mines the scientific literature for publications citing the use of individual antibodies, to generate a comprehensive searchable database of literature citations, together with suppliers, blog and news.

Establishing methods “fit for purpose” requires extensive validation and is a continuous process. Scientists, however, often claim that their methods are validated in a very casual way, often without explaining which guidelines were followed or any reference to experimental data to back up their claim. The validation process should start with a description of the purpose of the method, followed by method development and the definition of the performance characteristics. It continues with documentation of the methodology and the validation results. Once the method has been validated, it is crucial that the method is consistently executed as it was validated. The same materials should be used, and system suitability and method controls are applied with the same specifications as during the validation process. During the in-use phase of the methods, there is continuous monitoring to assure that the method still generates results in accordance with the performance characteristics as originally determined. Revalidation is needed if the method is changed, or has been out of use for a while, when it is applied for another material or for a new purpose. For this, the validated method is carefully documented in a procedure or a standard protocol often called a Standard Operating Procedure or ‘SOP’. The documentation is not limited to a general description of the methods, but preferably includes all relevant aspects that contribute to the performance of the method.

## Future Directions and Outstanding Questions

Validation of PD biomarker methods is a necessary component for obtaining high-quality PD biomarker data from clinical studies. For this purpose, FDA draft guidelines for the validation of quantitative PD biomarker methods have existed since 2013 [9]. In this light, the incomplete and sometimes total absence of validation for 87% of quantitative PD biomarker methods in phase 1-3 clinical cancer trials, published in the last 7 years, is quite disturbing. A possible explanation for this lack of validation may be that most quantitative biomarkers used in clinical trials originate from preclinical research in laboratories that are not used to working in accordance with good laboratory practice (GLP). GLP guidelines mandate full validation of quantitative bioanalytical drug methods. For quantitative biomarker methods these guidelines did not exist till the FDA recommended them in their 2013 draft BMV guideline, which became final in 2018 [9].

The FDA BMV 2018 guideline is, however, limited to recommendations for chromatographic and ligand binding method validation [9]. This may explain why we found that validation was most often lacking for quantitative FC biomarker methods [92]. These FC methods are used to quantify cell subpopulations and antigen expression per cell [93]. Quantitative FC methods are used in many clinical biomarker studies, and their importance is increasing [93]. Several factors complicate the validation of these FC methods [94]. One of the most important limitations is lack of standardization in methods, instrument setup, and quality controls. There is an urgent need to address these limitations by establishing internationally harmonized guidelines for quantitative FC biomarker methods used for clinical trials.

Currently, journals apparently do not mandate the use of properly validated quantitative PD methods for clinical studies. Investigators should, however, realize that quantitative PD methods used in clinical trials can only generate reliable and robust results if these methods are fully validated, reliable and robust. In that respect, it does not matter whether these methods are used for regulatory decision making, or for exploratory purposes. Full validation will increase the credibility of PD studies and may increase the discovery rate of qualified clinical PD biomarkers. For this purpose, we recommend that all quantitative PD methods used in clinical trials are fully validated as in the international guidelines for bioanalytical method validation [9].

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None

## Authors' Contributions

DP analyzed and interpreted the data. DP and JB

were major contributors in writing the manuscript. JB supervised the study, provided critical suggestions to the study. All authors read and approved the final manuscript.

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## Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Ethics Approval and Consent to Participate

Not applicable.

## Consent for Publication

Not applicable.

## Competing Interests

The authors declare that they have no competing interests.

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