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Review

Insights in the application of research-grade diagnostic kits for biomarker assessments in support of clinical drug development: Bioanalysis of circulating concentrations of soluble receptor activator of nuclear factor κB ligand

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ABSTRACT

Application of research-grade diagnostic kits in clinical drug development has grown commensurate with the increased interest in utilization of biomarkers as drug development tools. Since novel biomarkers are frequently macromolecular, immunoassay methodology comprises the 'technology-of-choice' for biomarker quantification. In particular, commercial research-grade immunoassay kits are appealing for use in biomarker quantification during clinical phase drug development because of their ready availability, ease of operation and perceived convenience. However, bioanalytical validation issues arise often during the application of commercial kits, as GLP regulatory-compliant application places greater demands on kit design and performance. In this review, we have used the receptor activator of nuclear factor κB ligand (RANKL) as a model system to offer some insights into the challenges that can be encountered in the application of 'research-grade' diagnostic kits in support of clinical drug development.

Currently only a few assays are available commercially for the determination of circulating concentrations of sRANKL. Of these, two immunoassay designs have been most often. The first design employs human osteoprotegerin to capture unbound sRANKL from serum and, thereby, provides a measure of circulating free concentrations. In contrast, the other common assay design first involves preincubation of serum samples with human osteoprotegerin to convert the free fraction of sRANKL to the osteoprotegerin-bound complex. The bound fraction is subsequently captured by an anti-osteoprotegerin antibody. In both immunoassay designs, detection is accomplished with an anti-sRANKL enzyme conjugation system. In this report we review these sRANKL immunoassay designs critically from the perspective of their potential suitability as drug development biomarker tools. In addition, analytical challenges relevant to the application of these 'research-grade' diagnostic kits for regulatory-compliant determination of sRANKL concentrations are discussed.

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Abbreviations: RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; sRANKL, soluble fraction of receptor activator of nuclear factor κB ligand; OPG, osteoprotegerin; hOPG, human form of osteoprotegerin; ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection.

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1. Introduction

Application of biomarkers is viewed widely within the pharmaceutical industry as a key strategy for improving the success rate and cost-effectiveness of rational drug development [1–3]. When applied successfully, biomarker data can be used to help select lead compounds, provide key safety and efficacy information, generate PK/PD models, confirm a drug's mechanism-of-action, serve as surrogates for clinical or mortality endpoints, aid in stratification of patients, and facilitate dose selection for pivotal registration trials. Despite their promise and broad application in recent years, successful biomarker testing and utilization remains a challenging endeavor [4,5]. In the worst case a biomarker strategy that is not planned and executed successfully can consume resources and contribute little towards increasing the efficiency of the drug development process. Consequently, attention has been focused over the past decade on strategies for improving both analytical and clinical validity to help ensure the likelihood that a potential biomarker(s) will add value during clinical drug development. There is an important distinction between these important validation concepts. Clinical validation, also referred to as qualification, is the evidentiary process of linking a biomarker with biology, pharmacology and/or clinical endpoints [1,4], whereas analytical validation is a formal process of evaluating an assay to ensure it provides reliable data [6]. Analytical validation is important, as issues herein can limit the quality and utility of biomarker data. Moreover, application of an assay that lacks analytical validity can potentially undermine successful demonstration of a pharmacodynamic effect during clinical investigation. Unlike assays used to conduct pharmacokinetic assessments of drugs [7], formal regulatory guidance is lacking for analytical validation of novel biomarker assays. A novel biomarker is defined as an analyte or activity that is measured by an *in vitro* assay that is not available as a routine clinical laboratory test [8,9]. Recently, a 'fit-for-purpose' validation strategy, which involves iterative method refinement, optimization and validation, was proposed as a means for maximizing the efficiency and cost-effectiveness of novel biomarker testing in drug development [10].

Three broad categories of assays are used to provide quantitative measurements of biomarkers in support of clinical drug development. These include novel biomarker assays developed within a pharmaceutical company, clinical-grade diagnostic kits and 'research-grade' diagnostic kits. Clinical-grade diagnostic kits are often FDA approved tests and performed on an instrument-based platform. In contrast, research-grade kits are not FDA approved, usually not instrument-based and often employ specialized technology or equipment. The topic of research-grade diagnostic kits warrants further discussion, as these kits are appealing for use because of their ready availability, ease of operation and perceived convenience. In addition, research-grade kits are nearly always first-to-market as quantitative assays for newly discovered biomarkers which make them attractive for supporting development of new innovative therapeutics. However, for numerous reasons application of research-grade diagnostic kits frequently poses a considerable challenge for biomarker analytical validation, especially if the intended purpose is to provide regulatory or GLP-compliant validation and clinical trial data. Some of the frequently encountered issues for regulatory-compliant utilization of research-grade diagnostic kits are outlined in Table 1. In this review, we have used the receptor activator of nuclear factor κB ligand (RANKL) as a model novel biomarker to offer insights into some of the scientific, regulatory and practical issues encountered in the application of research-grade diagnostic kits in support of clinical drug development. Commercial kits for this cytokine are demonstrative of why it is important to consider an assay's attributes from the perspective of 'suitable for its intended purpose' before selecting one for use as a drug development biomarker tool.

The receptor activator of nuclear factor κB ligand is an essential cytokine that serves as an important determinant for osteoclastogenesis and bone resorption [11,12]. The physiologic functions and

Table 1
Common issues for application of research diagnostic kits in clinical drug development.

- Reference material:
  - May not be representative of endogenous 'biologically active' form
  - Vendor–vendor variability
  - Often employ a secondary standard that is qualified against a primary standard
  - Lack of documentation for reference characterization, purity and stability
  - Lot-to-lot variability and lots changes not communicated
  - Limited availability
    - Complicates additional characterization
    - Precludes use in 'spike-recovery' experiments
- Kit components:
  - Kit and lot-to-lot variability in key components (*e.g.*, antibodies)
  - Dependent on manufacturer for continuous supply of critical components
  - Reagent volumes/supply is insufficient for automation
  - Kit calibrators
    - May differ from primary reference
    - Formulated in buffer (*i.e.*, lack matrix)
    - Too few for optimal curve-fitting
  - QC samples
    - Not included in kit
    - Formulated in buffer (*i.e.*, lack matrix)
    - Range is too limited
    - If included, may be identical to calibrators
- Kit analytical validation:
  - Conforms to kit manufacturer's specifications
  - Inconsistency among kit manufacturers
  - Validation information and documentation are incomplete
  - Assay specificity information is lacking
    - Antibody specificity is not reported
    - Cross-reactivity with endogenous analyte forms is not reported
  - Validation is not representative of GLP
    - Method acceptance criteria are not specified
    - Sensitivity is reported in terms of LOD not LLOQ
    - Validated range is not specified
    - Dilutional linearity information lacking
    - Selectivity data lacking (*e.g.*, impact of matrix and disease-state)
    - Biomarker matrix stability information is lacking
- Operational:
  - Different results are obtained by different manufacturers
  - Limited pre-analytical instructions for sample processing and handling
  - Run acceptance criteria are not specified
  - Biomarker range data are not reported for normal healthy adults and patients with disease
  - May not be suitable for monitoring changes in biomarker concentrations in some patient populations
  - May not be suitable for monitoring changes in biomarker concentrations after drug treatment
  - Study drug may cause interference and invalidate assay
  - Co-administered drugs may cause interference and invalidate assay
  - Inconsistency across labs in curve-fitting of nonlinear immunoassay data

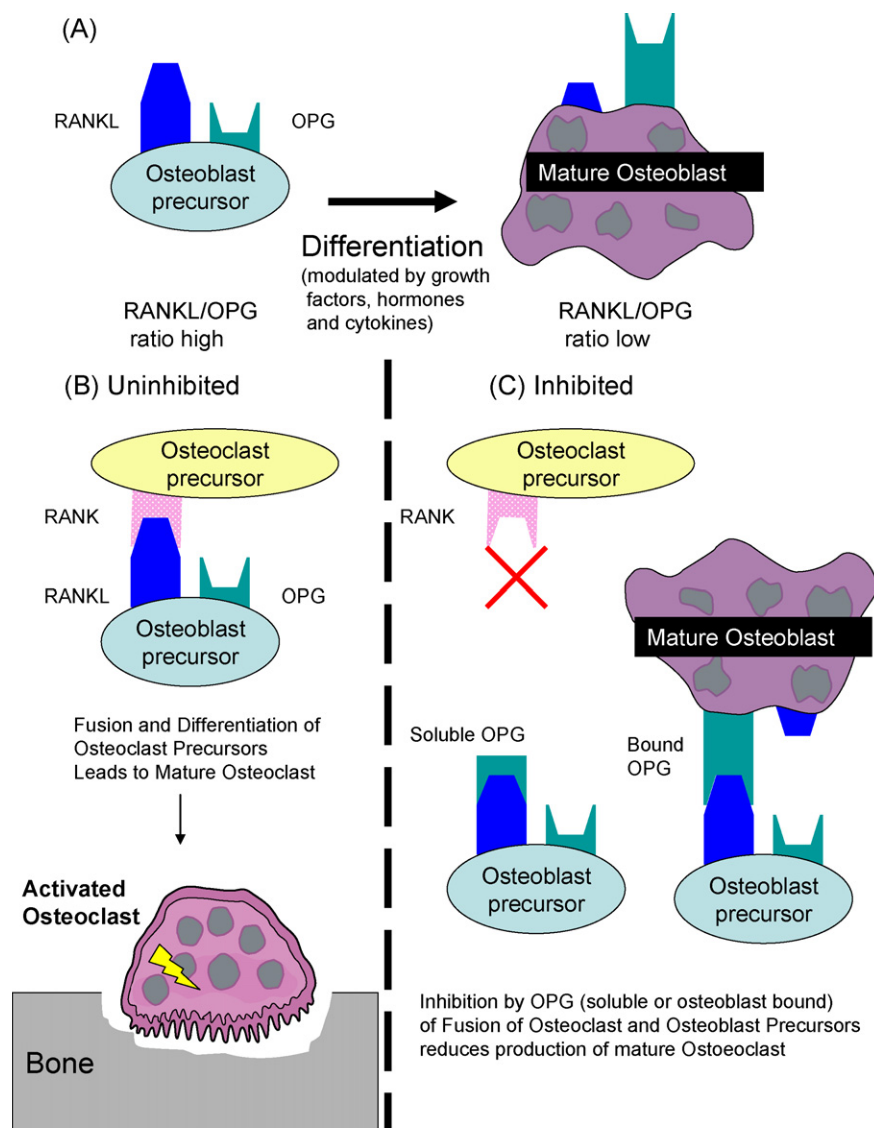

Fig. 1. Physiological interactions between RANKL, RANK and OPG in controlling osteoclast differentiation and maturation.

potential pathophysiologic effects of RANKL have been reviewed in a number of recent publications [13–15] and are depicted schematically in Fig. 1. Because of its known involvement in bone resorption and remodeling, RANKL has been an attractive target for drug development [16–20]. In brief, RANKL is a type II homotrimeric transmembrane protein with close homology to member ligands of the TNF superfamily. RANKL was discovered during the search for a ligand for osteoprotegerin (OPG) [12,14]. RANKL also exists in a secreted soluble form which is derived from the membrane form as the result of either proteolytic cleavage or by alternative splicing and binds to receptor activator of NF-κB (also referred to as RANK and Trance-R). The binding of RANKL to RANK on osteoclast precursors induces a signaling cascade leading to the differentiation and fusion of osteoclast precursor cells (Fig. 1B), while binding to mature osteoclasts triggers activation and survival (Fig. 1A) [21,22]. The biochemical effects of RANKL are counterbalanced by osteoprotegerin, a soluble neutralizing decoy receptor and member of the TNF receptor superfamily [12,14,23]. Through its decoy receptor activity by binding and neutralizing RANKL, OPG inhibits osteoclastogenesis, osteoclast activity and induces apoptosis (Fig. 1C) [24]. The high affinity interaction between RANKL and OPG has been exploited as the basis for establishment of assays to quantify circulating concentrations of sRANKL.

RANKL is present *in vivo* in different states with the membrane-bound form comprising the major form (Fig. 2) [11,12,25]. As such, the usefulness of measuring circulating soluble sRANKL as a drug development biomarker remains unclear [11,14,26,27]. In addition to its existence in both membrane-bound and soluble forms, RANKL is found in the circulation in either in a free form or complexed with OPG with the latter form predominating [12,14,23]. Furthermore, RANKL circulates as a 61 kDa homotrimer [24] and is found in tissues in three different isoforms [25]. Therefore it is not surprising that molecular heterogeneity is a key factor that complicates the determination of sRANKL by immunoassay methodology. For this reason an understanding of assay design and specificity are crucial for application of a 'research-grade' diagnostic kit and interpretation of resultant sRANKL biomarker data. Often as biochemical and physiological data accumulate for a novel biomarker, it is useful for the bioanalytical method to undergo an iterative process of optimization and validation to ensure the assay provides reliable data. As relevant information is gained, systematic changes to the analytical method may be warranted.

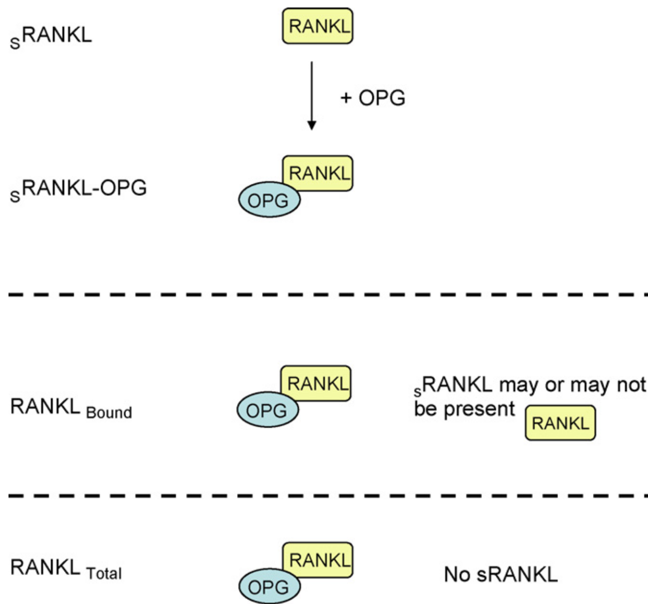

Fig. 2. Multiple endogenous forms of RANKL. RANKL exists in both a soluble (sRANKL) and membrane-bound forms with or without association with OPG. $RANKL_{total}$ is what is measured after hOPG is added to samples in excess to convert sRANKL to OPG/RANKL.

2. Immunoassay format, design and limitations

Currently all commercial immunoassays for sRANKL are variants of conventional ELISA methodology. In addition to the common ELISA format that employs anti-RANKL antibodies for both capture and detection [28,29], two alternate assay designs are used widely in commercial kits (Fig. 3). The important distinction between these ELISA designs concerns the different forms of sRANKL that are detected. Since Format 1 utilizes OPG for capturing sRANKL, it is only capable of detecting free forms of circulating sRANKL, *i.e.*, those not complexed with OPG. In contrast Format 2, which employs an anti-OPG capture antibody, detects the bound forms of circulating sRANKL *i.e.*, those complexed with OPG. A design variant of Format 2 for quantifying total circulating sRANKL involves preincubation of serum samples with recombinant hOPG to convert free sRANKL to OPG-bound sRANKL prior to antibody capture. Hypothetically, this modified approach would provide a measure of total circulating concentrations of sRANKL, *i.e.*, OPG-bound + free. Undoubtedly, design differences among commercial assay kits is one major factor that complicates interpretation of clinical data and confounds data comparison across studies when different assays are used for quantifying sRANKL.

3. Critical review of commercial sRANKL immunoassays

Currently only a limited number of immunoassays are available commercially for the quantitative determination of circulating sRANKL concentrations. All kits are categorized as research-grade diagnostics and include the disclaimer, '*for research purposes only*'. Characteristics of some commonly used commercial kits are summarized in Table 2. In the following sections, we review the major immunoassay designs for sRANKL with respect to the common issues for research-grade kits as outlined in Table 1. In addition, we attempt to highlight analytical performance characteristics and factors that may limit utility as biomarker tools for supporting clinical drug development.

One issue that is pertinent to all analytical designs is analyte heterogeneity and micro-heterogeneity. As noted previously,

Table 2
Commercially available sandwich immunoassays for determination of circulating concentrations of sRANKL.

	Kit manufacturer				
	Biomedica Gruppe	Immunodiagnostic systems (IDS)	Kamiya Biomedical Co.	Immundiagnostik/Apotech (co-developed)	Millipore
Website	www.bmgrp.com, www.alpco.com, www.bionetinc.com	www.idltd.com, www.idsinc.us.com	www.kamiyabiomedical.com	www.immundiagnostik.com, www.apotech.com	www.millipore.com/catalogue/item/hbn51k1rankl
Distributors (USA)	ALPCO Diagnostics BioNet Inc.	IDS US	–	ALPCO	MILLIPORE
Kit catalog #	04-BI-20422H	FS-03F1	KT-014	APO-54N-016-K101	HBN51K1RANKL
Analyte	Free sRANKL	Free sRANKL	Free sRANKL	Total sRANKL	Total sRANKL
ELISA format (Fig. 2)	#1	#1	#1	#2	NA
Capture	rDNA hOPG	rDNA hOPG	rDNA hOPG	Pab anti-OPG + hOPG	Anti-Human RANKL coated Luminex beads
Calibrators and matrix	? and buffer	? and buffer	? and buffer	rDNA sRANKL and buffer	rDNA sRANKL and human serum matrix
Calibrator range (pM)	1.25–10	1.25–10	0.38–6	~6–480	0.14–571 (assumed 35 kDa)
Calibrator range (pg/mL)	25–200	25–200	7.6–120	~37–30,000	5–20,000
Matrix and sample dilution	Serum/plasma and neat	Plasma and neat	Serum/plasma and neat	Serum/plasma and 1:10	Serum/plasma and 1:2
Detection (anti-sRANKL)	Biotinylated PAb	Biotinylated PAb	Biotinylated PAb	Biotinylated MOAb	Biotinylated Ab
Conjugate/substrate	Strepavidin-HRP/TMB	Strepavidin-HRP/TMB	Strepavidin-HRP/TMB	Strepavidin-HRP/TMB	Strepavidin-phycoerythrin

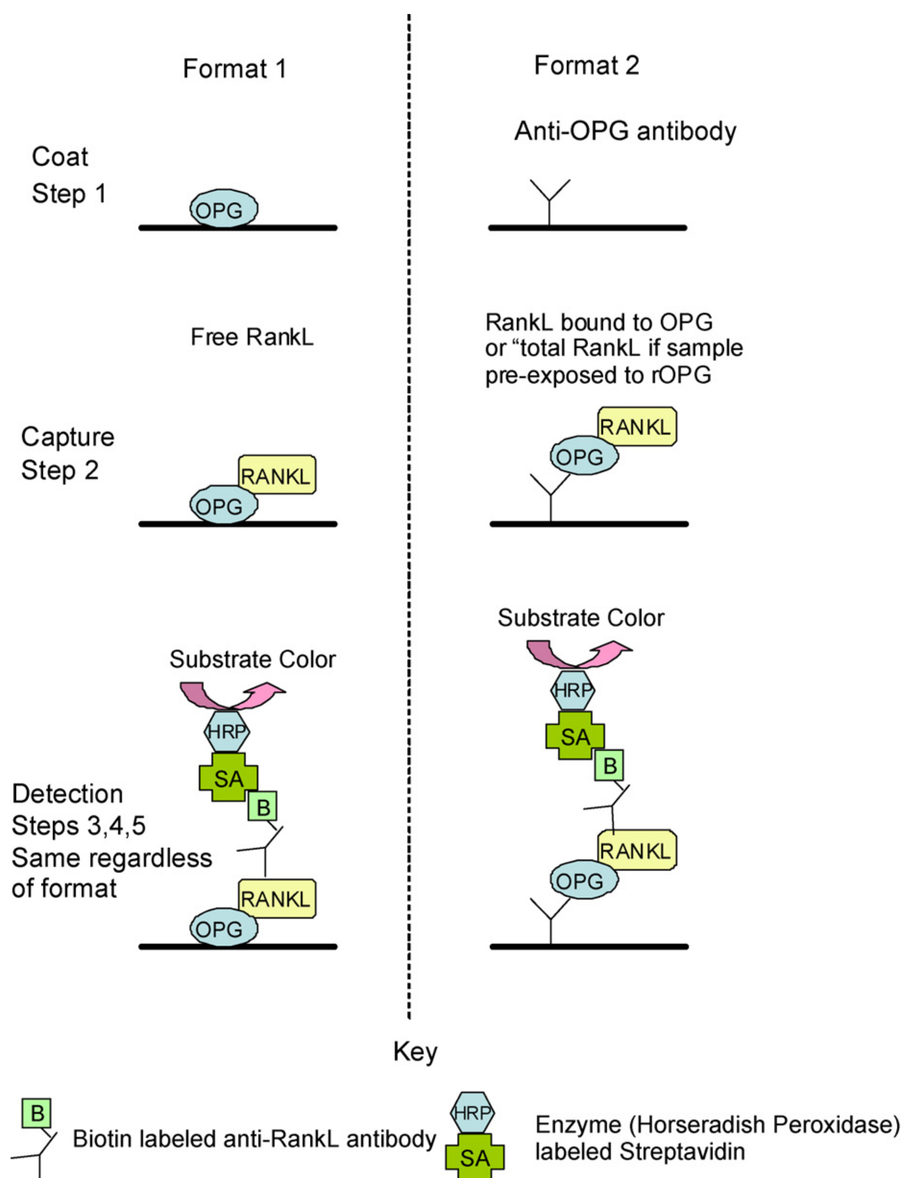

Fig. 3. ELISA formats for determination of RANKL. Format 1 measures free sRANKL, whereas Format 2 detects both $RANKL_{bound}$ and $RANKL_{total}$.

sRANKL is reported to be found *in vivo* in different multimeric forms [29]. With respect to commercial kits, multimeric forms of sRANKL will likely differ in their immunoreactivity vis-à-vis the reference standard which can lead to differences in both potency and parallelism. Parallelism of the calibration (standard) curve and the concentration–response for analyte in a test sample is a necessary condition for validity of an analytical result when the mean response is defined as a function of log dose (*e.g.*, the sigmoidal concentration–response relationships observed in immunoassays) [30].

A similar issue concerns the nature of the reference material used as the calibration standard in commercial biomarker kits (Table 1). Since this rDNA-derived material is not fully representative of the sRANKL forms found *in vivo* and differs across commercial kits and is also subject to lot-to-lot variability, it is not surprising that discordance would exist for reference calibrators across commercial kits. This was confirmed recently by demonstrating that a calibration standard from one commercial sRANKL assay did not to display comparable immunoreactivity/potency when tested in a different commercial kit (Jean Lee communication, unpublished data from Amgen). Thus, the unknown heterogeneity and micro-heterogeneity in circulating sRANKL in combination with the lack of availability of a well-characterized uniform reference standard are important factors that contribute to the discordance in measured serum concentrations found among commercial kits. It should, therefore, not be too surprising that it is often difficult to combine sRANKL data across different commercial research-grade diagnostic kits. In this regard, it is likely better to compare relative baseline changes in sRANKL concentrations as opposed to making comparisons of absolute serum concentrations.

3.1. Conventional ELISAs for sRANKL

A few conventional ELISA methods have been reported for the quantitative determination of sRANKL [28,29,31]. ELISA reagents and kits are available commercially from several sources, such as R&D Systems (Minn., MN), PeproTech (Rocky Hill, NJ) and MILLIPORE (St Charles, MO). While the majority of commercial kits exploit the high affinity interaction between OPG and sRANKL as their basis for quantification, conventional sandwich ELISAs

employ anti-RANKL antibodies for both analyte capture and detection. Interestingly the mean serum concentrations determined by conventional ELISA are at least 10–100-times greater than those reported for free sRANKL (Fig. 3, Format 1). The higher levels suggest that OPG binding does not completely mask the binding epitopes for the anti-sRANKL antibodies. From a method validity perspective, this finding raises questions about assay specificity, relative potency and parallelism of the various circulating forms of sRANKL, and the suitability of the recombinant calibration standard. Thus, an understanding of the cross-reactivity of circulating forms of sRANKL with antibodies in conventional ELISAs is crucial for understanding what constitutes sRANKL immunoreactivity in serum. In addition, other issues related to the reference standard as described in Table 1 are likely to be relevant to this ELISA format. Presently, sparse published information necessitates that conventional sRANKL ELISAs undergo more extensive analytical validation to understand their attributes and assess their appropriateness as biomarker tools for performing regulatory-compliant determinations in support of clinical drug development.

3.2. Immunoassays based on OPG capture

The analytical strategy involving use of hOPG to capture sRANKL was the first reported immunoassay design for quantitative determination of sRANKL [32,33]. This design is the basis for several commercial kits, including one from Biomedica Gruppe and others (Table 2). This assay design has been used widely in investigations of sRANKL [34–38]. As noted previously, this immunoassay format only detects free forms of sRANKL (Fig. 3, Format 1). In addition to issues already noted for the reference material, the kits for free sRANKL are characterized by numerous issues that are common to research-grade diagnostic kits (Table 1). The detection antibody is typically polyclonal and prone to lot-to-lot variation. Kit standards are formulated in buffer and the number of standard is too few for optimal application of a weighted 4/5-parameter logistic model, the preferred curve-fitting algorithm for regulatory-compliant bioanalysis [7,30]. As commonly seen with research-grade diagnostic kits, the Biomedica Gruppe kit lacks QC samples, information regarding Pre-study method validation and In-study run acceptance criteria are absent.

Since OPG-bound forms of sRANKL are known to predominate in the circulation [12,14,15], it is not surprising that serum concentrations of free sRANKL measured by assay Format 1 are low relative to others. In 2004, Schett et al. [35] used the Biomedica kit to quantify free sRANKL concentrations in 906 individuals, reported an overall median concentration of 1 pmol/L (23 pg/mL), and concluded that individuals in the low tertile (median of 0.6 pmol/L) were at increased risk for nontraumatic fractures. A review of representative published data from 10 studies from 2003 to 2007 that used the Biomedica kit yielded a mean serum sRANKL concentration in normal adults of about 0.7 pM or 16.2 pg/mL, assuming a molecular weight of 23 kDa. In a number of studies investigators reported that a substantial fraction of their measured values were below the assay's lower limit of detection (LOD) (Fig. 4). In addition in at least one study, values below the LOD were excluded to facilitate calculation of the mean sRANKL concentration in normal subjects [27]. Exclusion of values below the LOD would yield a mean reported result that is biased high relative to the uncensored value. These investigators concluded that measurement of the free fraction of sRANKL was of limited practical value in the study of postmenopausal osteoporosis, as a large percentage of their serum results were very low, near the lowest assay calibrator and regarded as assay noise [27]. As described in the Biomedica kit brochure, the median expected values for normal healthy females and males are 7.4 and 9.2 pg/mL, respectively [39]. From a regulatory-compliant

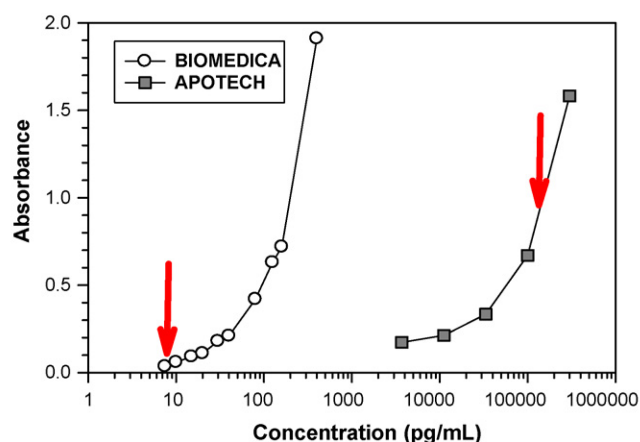

Fig. 4. Comparison of typical measured concentrations of free (Biomedica Gruppe) and total (Apotech) circulating sRANKL (shown in red) determined by different commercial kits relative to their standard curve calibrators. [*The calibration curve range of the Apotech kit was adjusted to account for the required 1:10 dilution of test samples.*] Note, normal circulating plasma concentrations of sRANKL (*e.g.*, typical concentration across numerous published studies) determined with the free ELISA occur near the lowest calibrator.

immunoassay validation perspective, results obtained near the limit of detection are inherently less reliable and more variable due to the 'flat' nature of the sigmoidal concentration–response relationship in this region (Fig. 4). In addition it is not advisable to employ extrapolation as a means to estimate concentrations of unknown test samples that are below the lowest calibrator [30]. Thus, data indicating that serum concentrations of free sRANKL in normal subjects are low and near the LOD present a substantial operational challenge for the current generation of immunoassays kits based on OPG capture. These data indicate that this immunoassay design is of limited value for providing reliable biomarker data to support clinical drug development. This would be particularly true, if the therapeutic intervention were anticipated to cause a decrease in circulating concentrations of sRANKL.

3.3. Immunoassays based on anti-OPG capture

Investigations employing sRANKL assays based on anti-OPG capture have been reported far less frequently than those for free sRANKL [40–43]. This is a consequence of the earlier availability of commercial kits for free sRANKL, including the Biomedica kit and others. It also reflects the progression of rational assay design, as additional information has accumulated regarding this putative biomarker. Recently, a co-developed kit became available from Immundiagnostik AG/Apotech (Table 2) [44]. The novel aspect of this ELISA involves preincubation of a diluted serum sample with OPG to convert free sRANKL to the OPG-bound form prior to capture by the anti-hOPG antibody. As noted previously, this approach provides a measure of total circulating concentrations of sRANKL, *i.e.*, OPG-bound + free. The striking difference in detectable concentrations between free and total circulating sRANKL can be inferred from a report by Hofbauer et al. [40]. In this study serum sRANKL concentrations were determined in healthy females (n = 38) by both the Biomedica (free) and Apotech (total) kits. The molar ratio of measured concentrations was on the order of 15,000-times greater for total sRANKL to those of free sRANKL. In 2008 D'Amelio et al. [43] reported that the mean total sRANKL concentration in 38 women with postmenopausal osteoporosis was approximately 300 ng/mL (∼4800 pM) or roughly 7000-times higher than the estimated mean serum concentration of free sRANKL determined by the Biomedica kit.

From a regulatory-compliant immunoanalytic perspective, the measurement of total sRANKL offers a number of potential advantages. First, the serum levels are markedly higher since the bound fraction comprises approximately 99% of circulating sRANKL. Second, the higher serum concentrations necessitate a 1/10 sample dilution which is beneficial for reducing assay matrix interference. Third, serum concentrations correspond to a region of the standard curve that provides superior accuracy and precision (Fig. 4). Despite the broader range of specificity of the Apotech kit for detection of circulating sRANKL forms, the discordance in serum concentrations is large and comparable to the variability in concentrations reported with the Biomedica ELISA. This observation suggests that other analytical factors are likely important in the performance of this commercial kit. In addition, the Apotech kit is characterized by a number of the issues noted in Table 1 that commonly impact application of research-grade diagnostic kits. The kit's calibrators are formulated in buffer and the number is too few to provide optimal curve-fitting. The manufacturer recommends, but does not provide QC samples and normal reference range information is lacking. Even though some method validation data are reported, the details are sparse, criteria are lacking and the design conformed to a manufacturer's specifications. In conclusion, immunoassays for total sRANKL that employ anti-hOPG antibodies for capture offer an attractive alternative to commercial kits for free sRANKL. However, the suggestion by Hofbauer et al. [40] that a statistically significant positive correlation exists between free and total serum levels of sRANKL is difficult to substantiate, since the free levels are low and in an unreliable region of the standard curve. Thus, important questions remain concerning the value of total sRANKL as a drug development biomarker. In spite of the attractiveness of measuring total circulating concentrations of sRANKL, additional bioanalytical work is needed to understand the important underlying pre-analytical and analytical factors and confirm its validity before this measurement finds wide application as a routine drug development biomarker tool.

4. Discussion

Prior to application of a 'research-grade' immunoassay kit in support of clinical drug development, careful evaluation is needed to characterize its analytical performance and identify sources of measurement bias and variability to ensure its suitability for providing reliable biomarker data. Analytical issues often limit the utility of commercial kits for application as biomarker tools, especially when regulatory-compliant measurements are needed. Despite being innovative and often first-to-market for new analytes, these immunoassay kits are developed as 'discovery-grade' research tools and, therefore, typically lack important analytical attributes that make them suited as clinical drug development biomarker tools. Common issues that complicate application of research-grade diagnostic kits for supporting clinical drug development are listed in Table 1. For this manuscript we conducted a detailed literature review of currently used research diagnostic kits for the determination of circulating concentrations of sRANKL to demonstrate these principles. It is clear that there is still much to learn about sRANKL measurements and what underlying factors are important for providing reliable information of this putative biomarker. More investigation is needed to understand whether free and total sRANKL measurements are useful as biomarker tools for predicting disease activity and/or drug pharmacodynamics.

Undoubtedly differences in ELISA design, antibody specificity, and analyte heterogeneity and micro-heterogeneity are important factors for the discordance in sRANKL measurements found currently among different immunoassays. This underscores the importance for need to understand antibody specificity and analyte heterogeneity in rational assay design. Despite early introduction and wide application by investigators to evaluate the diagnostic utility of sRANKL measurements, published information suggests currently available assays for free sRANKL are of limited value for providing reliable measurements at physiologically relevant concentrations and as biomarker tools to support clinical drug development. Numerous publications of free sRANKL kits are demonstrative of the inherent limitation for application of a research-grade diagnostic kit that lacks adequate sensitivity in a regulatory-compliant manner. This would be especially true in clinical paradigms where the therapeutic intervention is expected to result in a decrease in circulating concentrations of the potential biomarker.

If research-grade immunoassays for free sRANKL are perceived to be useful clinically as a drug development biomarker tools, then new strategies are needed to overcome their analytical limitation of inadequate sensitivity. Some potential solutions include employing improved antibodies with higher affinity and/or utilization of more sensitive detection methods, such as fluorescence or luminescence. This was demonstrated by the recent availability of a next generation sRANKL method, Immunodiagnostic systems (IDS) recently introduced a new kit, *ampli sRANKL human ELISA*, FS-04F1, in which a tetrazolium redox detection system was introduced for increased sensitivity. The calibrator range (0.125–2.0 pM) in this modified kit is about 1/10 the concentrations in the first generation assay with mean reported concentrations for males ($n = 394$) and females ($n = 635$) of 7.5 and 9.2 pg/mL, respectively.

Unless a strong justification can be made for quantifying free sRANKL as a drug development biomarker, the total ELISA employing anti-OPG capture represents the logical alternative as a commercial kit for supporting clinical investigations. This is because too little information is available now concerning the potential utility of a conventional ELISA approach for providing reliable sRANKL data. The total ELISA design would seem to offer a number of advantages for overcoming the limitations of free sRANKL measurements and be better suited for application in a regulatory-compliant manner. These include detection of markedly higher circulating concentrations, ample sensitivity, inclusion of sample dilution to reduce matrix interference, and detection of both free and OPG-bound forms of sRANKL. However, limited information has been reported to date with the total ELISA. Hence, more detailed assessment is needed to evaluate the utility of total sRANKL measurements before this assay design can be viewed as a valid biomarker tool for supporting clinical drug development.

In summary, in this review we have attempted to use measurements of circulating sRANKL as a model system to highlight issues pertaining to the application of 'research-grade' diagnostic kits as biomarker tools for supporting clinical drug development. To date much clinical data have been published for sRANKL using 'research-grade' diagnostic kits without systematic evaluation of their attributes and rigorous analytical validation. This confounds understanding the sources of analytical variability, the causes for discordant results across different commercial kits and precludes comparison of results across different assays. This model is demonstrative of the need for *a priori* consideration of the analytical requirements and the attributes of a commercial kit before implementation as a tool for biomarker quantification in support of clinical drug development.

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