Agreement in assessment of infliximab and adalimumab levels in rheumatoid arthritis: interlaboratory and interassay comparison

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Abstract

Objective

Infliximab (IFX) and adalimumab (ADL) drug levels and anti-drug antibodies (ADA) are assessed using a variety of techniques, therefore, results cannot accurately be compared for clinical purposes. The aim of this study was to test two infliximab (IFX) and adalimumab (ADL) ELISA versions, for drug levels and ADA, to see whether they yield similar results.

Methods

ELISA versions [Promonitor® IFX R1 and R2 (V.1), Promonitor® IFX and Anti-IFX (V.2); Promonitor® ADL R1 and R2 (V.1), Promonitor® ADL and Anti-ADL (V.2) kits (Progenika Biopharma, Spain)] were used to measure drug levels and ADA in IFX (n=24) and ADL (n=24) rheumatoid arthritis-treated patients in three independent laboratories. Quantitative and qualitative agreements were evaluated using intraclass correlation coefficients (ICC), and Cohen's Kappa (κ) respectively. The Bland-Altman plots assessed differences between V.1 and V.2.

Results

Interlaboratory agreement (ICC/κ) with V.1 was poor for IFX (0.66/0.62) and ADL (0.69/0.52) drug levels; meanwhile, high agreement was found with V.2 for IFX (0.98/0.95) and ADL (0.094/1.00). Comparison between V.1 and V.2 in each laboratory resulted in systematically higher values in V.2 than in V.1 and poor agreement (ICC/κ ranges) for IFX (0.12–0.7/ 0.19–0.42) and ADL (0.69–0.89 /0.50–0.73).

Conclusion

Qualitative measurements result in better agreement, as evidenced in our study. Greater agreement in V.2 compared with V.1 for IFX and ADL levels could be due to a better tune up. Further studies are required to standardise methods to establish therapeutic reference ranges.

Key words

enzyme-linked immunosorbent assay, interlaboratory comparison, anti-TNF, agreement, infliximab, adalimumab, anti-drug antibodies, rheumatoid arthritis

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Introduction

The introduction of anti-tumour necrosis factor (TNF) drugs has revolutionised the treatment of rheumatic diseases (1-3), spondyloarthropathies (SpA) (4, 5), inflammatory bowel conditions (6) and psoriasis (7). Nevertheless, a substantial number of patients either fail to respond or lose response over time. Prevalence of non-response varies from 10–40% (8-13).

One of the stronger hypotheses to explain the lack of response, secondary loss of efficacy to anti-TNF treatment agents or both, claims that antibodies (ABs) are produced which neutralise the anti-TNF drug pathways (14, 15). Recent studies have shown that the presence of ABs against anti-TNF agents infliximab (IFX, Remicade®, Centocor Ortho Biotech Inc., Malvern, PA, EE.UU) and adalimumab (ADL, Humira®, Abbott Laboratories, North Chicago, Illinois, EE.UU) seems to correlate with low drug levels (16), loss of efficacy ergo adverse effects (17-19). However, to date, the presence of anti-drug Abs (ADA) does not fully explain the lack /loss of response to treatment. The exact role of these antibodies is not yet fully understood.

In order to establish the aforementioned drug level and ADA relationship, there are two widely-used methods. Firstly, there is the enzyme-linked immunosorbent assay (ELISA), which is more user-friendly and enjoys greater universal access, and then there is the radioimmunoassay (RIA) (20), which wins in terms of sensitivity but loses on cost and requires advanced laboratory facilities. There are, however, inconsistencies between the two owing to technical differences which can result in analytical discrepancies. This has raised concerns about subsequent application in clinical practice (21-23). Understanding the possible causes of these discrepancies will therefore aid our ability to improve ELISA measurements (23-25). Furthermore, there is growing clinical interest in the measurement of anti-TNF drug levels as part of future intervention strategies, so the need for standardised analytical procedures is paramount (26, 27).

Over recent years, there have been several clinical publications addressing this issue, but the heterogeneous nature of the trials and techniques has made it difficult to compare study results and establish the clinical significance of results. Therefore, the aim of the present study was to determine the inter-laboratory and inter-assay agreement for IFX and ADL levels and determine whether qualitative or quantitative analysis of these results are reproducible.

Materials and methods

Patient selection

Three Spanish laboratories with distinguished pedigree in rheumatology and immunology (Lab1, Lab2 and Lab3) located in Madrid, Alicante and Barcelona, respectively, participated in this study. IFX-treated patients (n=24) were included from Gregorio Marañón University General Hospital and ADL-treated patients (n=24) from Marina Baixa Hospital, all after signing the informed consent. For IFX levels and ADA (V.2) only 23 patients were evaluated. Inclusion criteria were to have been diagnosed with RA under the American College of Rheumatology (ACR) 1987 criteria (28) and to have been undergoing standard first line biological drug therapy for at least 12 months. This cross-sectional observational study was approved by the Medical Ethics Committees of both the Gregorio Marañón University General and Hospital Marina Baixa hospitals.

Samples preparation and submission

Serum samples were taken immediately prior to drug administration in each day care unit. After blood collection, the samples were centrifuged at 2500 rpm for 15 min and the serum samples then divided into three aliquots (1 ml each) and coded from 1 to 24 for IFX and ADL. Samples were then frozen on dry ice and distributed to all three participating laboratories. All samples were evaluated in a blinded way for drug levels and ADA.

Determination of IFX and ADL levels and ADA concentration

Serum IFX and ADL levels and ADA were measured using two different ELISA assays [Promonitor® IFX R1 and R2

(V.1), Promonitor® IFX and Anti-IFX (V.2) and Promonitor® ADL R1 and R2 (V.1), Promonitor® ADL and Anti-ADL (V.2) kits respectively (Progenika Biopharma, Spain)], strictly following the the manufacturer's instructions. All kits had identical lot numbers. The participant laboratories analysed primary data and produced results through implementation of their own standard procedures and statistical calculation models. The serum samples were sequentially diluted and a calibrated curve was drawn using the standard solution included in each kit. Absorbances (OD) were analysed using the Analysis Software Solutions (MyAssays, Ltd 2009). Cut-off values together with interpretation were described by the manufacturer for each ELISA version and assay; the main technical differences are detailed in Table I. Both assays were certified to be in compliance with FDA (Federal Drug Administration) and EMA guidelines (European Medicament Agency).

Statistical analysis

Quantitative as well as qualitative agreements were evaluated. Qualitative agreement after recoding data was calculated in line with the manufacturer's cut-off values. Quantitative results were summarised graphically using boxplots showing median, first and third quartiles, minimum and maximum. The Wilcoxon test was used to compare results of both versions in each laboratory.

We evaluated interlaboratory agreement for ELISA V1 and V2 as well as IFX and ADL levels and ADA concentrations. For the ADL and IFX agreement, in both versions, we used the intraclass correlation coefficient (ICC), with ranges from 0.7 to 0.89 meaning a questionable agreement; >0.9, high agreement with 1 (maximum value) meaning excellent agreement (29). After recoding, the qualitative agreement for ADL and IFX was determined by calculating the overall agreement of samples and Cohen's weighted kappa (κ). This coefficient is defined as a poor agreement when <0.2; fair when 0.21 to 0.4, moderate when 0.41 to 0.6, good when 0.61 to 0.8, and very good when 0.81 to 1 (30).

Interassay agreement between V.1 and V.2 for IFX and ADL levels and ADA concentrations in each laboratory was assessed. Quantitative results were evaluated by ICC, and qualitative results through Cohen's unweighted kappa (κ). Graphical analysis plotted the difference between the two versions, against their mean for each sample (Bland-Altman Plots). Mean difference (bias) and its 95% confidence interval were computed. Limits of agreement (LOA) were defined as mean difference ± 2 standard deviations of difference (LOA = bias±SDD). Ninety-five percent of differences are expected to lie between LOA. Individual discrepancies between methods may be observed, as well as systematic bias or trend in differences related to mean values (31).

P-values <0.05 were considered significant. Statistical analysis was performed by using Prism v 5.0 (Graph Pad Software Inc., La Jolla, CA, USA) and STATA-9 (Data Analysis and Statistical Software Stata Corp LP, TX, USA).

Results

Quantitative results are summarised graphically in boxplots showing median, first and third quartiles, minimum and maximum. All laboratories reported higher IFX levels in V.2 than V.1 (p<0.0005; in all comparisons), where-

Table I. Technical characteristics of V.1 and V.2 commercial ELISA assays.

	ELISA Version 1		ELISA Version 2	
Technical characteristics	Drug levels	Anti-drug antibodies	Drug levels	Anti-drug antibodies
Sample volume and dilutions	10 ul (6 dilutions)	10 ul (6 dilutions)	100 ul (2 dilutions)	200 ul (1 dilution)
Calibration curve points	10	10	6	6
Microtiter plate coated	No	No	Yes	Yes
Microwell volume	50 ul	50 ul	100 ul	100 ul
Reactives	Concentrate	Concentrate	Pre-diluted	Pre-diluted
Shaking	Yes	Yes	No	No
Positive and negative controls	No	No	Yes	Yes
Enzyme-labelled antibody	Biotin	Biotin	Streptavidin-Horseradish Peroxidase	Streptavidin-Horseradish Peroxidase
Processing time	6 hours	6 hours	2.5 hours	2.5 hours
Calibration range	IFX: 0 - 0.24 µg/ml ADL: 0 - 0.10 µg/ml	IFX: 0 - 40 AU/ml ADL: 0 - 100 AU/ml	IFX: 1.0 - 0.072 µg/ml ADL: 1.25 - 0.060 µg/ml	IFX: 2.0 - 144 AU/ml ADL: 3.13 - 200 AU/ml
Cut-off values for IFX	Negative: <0.053 µg/ml Low positive:0.053 - 1.5 µg/ml Positive: >1.5 µg/ml	Positive: >37 AU/ml	Negative : <0.035 µg/ml Low positive:0.035 - 1.5 µg/ml Positive: >1.5 µg/ml	Positive: >2 AU/ml
Cut-off values for ADL	Negative: <0.004 - 1.5 µg/ml Low positive: 0.004 - 0.8 µg/ml Positive: >0.8 µg/ml	Positive: >32 AU/ml	IFX: 0.053 - 1.5 µg /ml ADL: 0.004 - 0.8 µg /ml	Positive: >3.5 AU/ml
Cut-off values interpretation	Negative: negative Low positive: subtherapeutic Positive: therapeutic	–	Negative: negative Low positive: subtherapeutic Positive: therapeutic	–

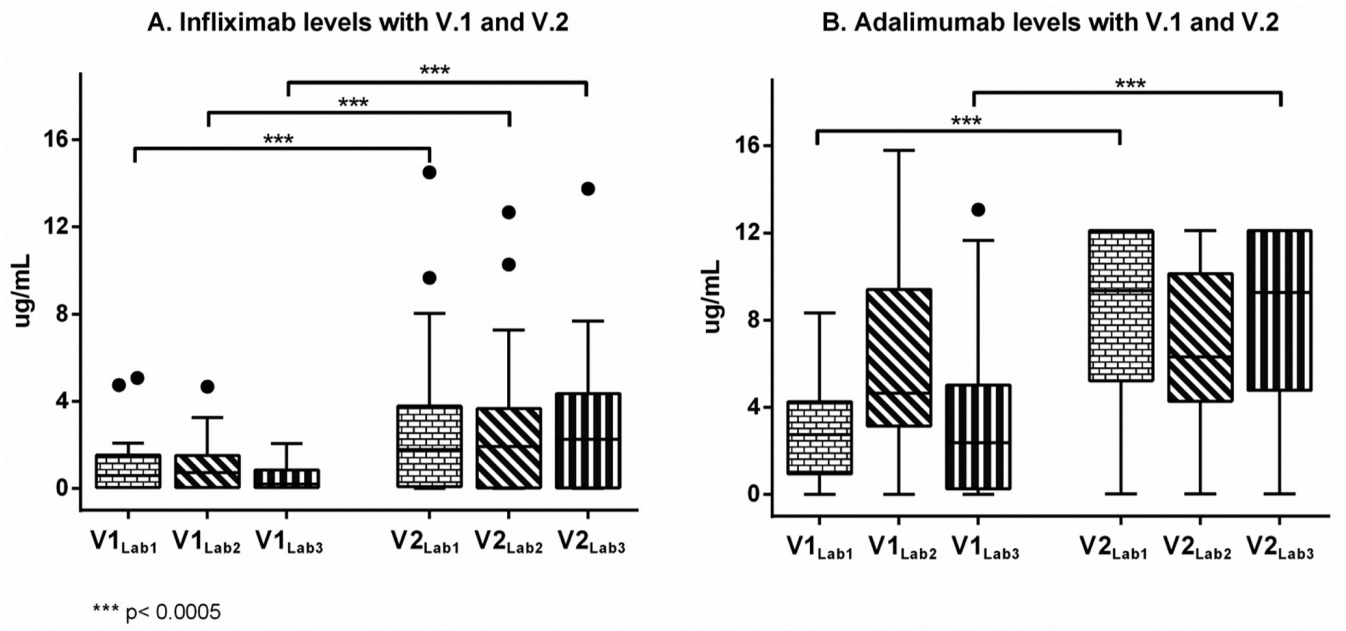

Fig. 1. A-B. Box plots show the median, quartiles and minimum and maximum values for IFX and ADL.

as Labs 1 and 3 reported higher ADL levels in V.2 than V.1 (p<0.0005; in both), but differences were not significant in Lab 2 (p=0.130), as evidenced in Figures 1 A and B.

V1 showed less overall agreement than V2 in terms of qualitative results for IFX and ADL levels. All laboratories had a better agreement, when a result was given as negative. Contrary to this, most disagreements occurred when a result was given as low positive or positive (Table II).

Regarding ADA concentration for IFX and ADL, only 5 and 3 samples respectively showed up positive in all laboratories; we consider this sample size too small to evaluate agreement between laboratories or ELISA versions.

V1 Drug level measurement between laboratories

a) IFX levels

Comparisons of quantitative results between laboratories using the ICC coefficient indicated a poor agreement (0.66, p=0.006). Sample concordance for IFX levels according to the manufacturers' cut-off values were 63% (15/24) with good qualitative κ coefficient agreement (0.62, p<0.0005) (Table III).

b) ADL levels

The quantitative agreement was poor (ICC=0.69, p<0.0005), while qualitative sample concordance was 71% (17/24) with a moderate agreement (κ=0.52, p<0.0005) (Table III).

V2 Drug level measurement between laboratories

c) IFX levels

Quantitative agreement between laboratories was high (ICC=0.98, p<0.0005) as was qualitative agreement in results (κ=0.95, p<0.0005), with a sample concordance of 87% (20/23) (Table III).

d) ADL levels

Quantitatively, the agreement was high (ICC=0.94, p<0.0005) and the qualitative agreement was excellent (κ=1, p<0.0005) as was sample concordance (100%, 24/24) (Table III).

Comparison between V.1 and V.2 for each laboratory

a) IFX levels

Quantitative agreement between versions for Lab1 was questionable (ICC=0.70, p=0.018) and for Lab2 and Lab3, it was poor (ICC=0.64, p=0.032; ICC=0.12, p=0.4). Qualitative agreement for Lab1 and Lab2 was moderate (κ=0.40, p=0.032; κ=0.42, p=0.002) and for Lab3, it was poor (κ=0.19, p=0.07). Sample concordance was 59% for Lab1, 50% for Lab2 and 38% for Lab3 (Table IV).

For IFX levels we found the same systematic bias for all three laboratories: the higher the mean of two observations, the higher was its difference. In addition, the mean difference observed in all laboratories did not include the zero in the confidence interval (Lab1: 1.93, 95% CI 0.75–3.11, Lab2: 1.84, 95% CI 0.72–2.95 and Lab3: 2.34, 95% CI 0.96–3.71), showing that V.2 values were consistently higher with respect to V.1. The limits of agreement in all laboratories were too large for us to draw any solid clinical conclusions (LOA Lab1:-3.3–7.2, Lab2: 3.2–6.9, and Lab3: -3.9–8.6) (Fig. 2 A-C).

b) ADL levels

Quantitative agreement between versions for Lab1 and Lab3 was poor (ICC=0.69, p=0.007; ICC=0.69, p=0.007); for Lab2, it was high (ICC=0.89, p<0.0005), while qualitative agreement for Lab1 and Lab2 was good (κ=0.64, p<0.0005; κ=0.73, p<0.0005) and for Lab3, it was moderate (κ=0.50, *p<0.0005*). The sample concordance was 88% for Lab1, 92% for Lab2 and 83% for Lab3 (Table IV).

For ADL levels, bias was not as clear as for IFX, but the higher differences noted mainly in Lab2 can be explained by the higher means (0.51, 95%CI -0.37–1.38). Moreover, Labs 1 and 3 showed some lineal regularity concerning the higher mean values of ADL which

Table II. Summary comparison of qualitative results for IFX and ADL levels with V.1 and V.2.

Results between Labs		N=	All negative	All low positive	All positive	1 neg. & 2 low pos.	2 neg. & 1 low pos.	1 neg. & 2 pos.	1 low pos. & 2 pos.	2 low pos. & 1 pos.	Overall agreement
Drug Level	IFX V1	24	8	6	1	1	0	1	5	2	63% (15)
	IFX V2	23	5	3	12	1	0	0	1	0	87% (20)
	ADL V1	24	3	0	14	0	1	0	5	1	71% (17)
	ADL V2	24	3	1	20	0	0	0	0	0	100% (24)

IFX: Infliximab; ADL: Adalimumab; neg.: negative; pos.: positive.

Table III. Qualitative and quantitative agreement for IFX and ADL levels.

Anti-TNF	Version	Quantitative Agreement				Qualitative Agreement			
		n	ICC	95% CI	*p*	n	Kappa	*p*	Overall agreement
IFX	V1	16	0.66	0.21 - 0.87	0.006	24	0.62	<0.0005	63%
	V2	17	0.98	0.97 - 0.99	<0.0005	22	0.95	<0.0005	87%*
ADL	V1	20	0.69	0.35 - 0.87	<0.0005	24	0.52	<0.0005	71%
	V2	21	0.94	0.87 - 0.97	<0.0005	24	1.0	<0.0005	100%

IFX: infliximab; ADL: adalimumab; *: without Lab1 two samples; Kappa: Cohen's Kappa coefficient; ICC: intraclass correlation coefficient; CI: 95% confidence interval.

Table IV. Comparison between V. 1 and V. 2 for each laboratory with kappa and ICC coefficients.

Anti-TNF	Lab	Quantitative Agreement				Qualitative Agreement			
		n	ICC	95% CI	*p*	n	Kappa	*p*	Overall agreement
IFX	Lab1	14	0.703	0.075 - 0.905	0.018	22	0.407	0.002	59%*
	Lab2	15	0.644	-0.061- 0.880	0.032	23	0.422	0.002	50%
	Lab3	13	0.128	-1.856 - 0.734	0.408	23	0.191	0.07	38%
ADL	Lab1	20	0.690	0.216 - 0.877	0.007	24	0.644	<0.0005	88%
	Lab2	20	0.891	0.723 - 0.957	<0.0005	24	0.738	<0.0005	92%
	Lab3	21	0.696	0.250 - 0.876	0.007	24	0.503	<0.0005	83%

IFX: infliximab; ADL: adalimumab; *: without two samples; Kappa: Cohen's kappa coefficient; ICC: intraclass correlation coefficient; CI: 95% confidence interval.

departs from a random distribution of differences (4.93, 95%CI 3.56–6.3 and 4.54, 95%CI 3–6.08, respectively). The limits of agreement in Lab1 and Lab3 were too large for us to draw any solid clinical conclusion when compared to Lab2 (LOA Lab1: -1.43–11.3 and Lab3: -2.61–11.7) (Fig. 2 D-F).

Discussion

The results from this study provide strong evidence that Elisa V2 yields more consistent quantitative and qualitative results when measuring ADL and IFX levels. To draw this conclusion, we tested both versions in three separate laboratories and analysed the results. We also inquired as to whether quantitative or qualitative results yield greater agreement.

When we compared quantitative measurements, we observed how Bland-Altman plots displayed a systematic bias for IFX and a systematic overestimation for ADL levels for V.2 *versus* V.1 in all laboratories. Qualitative agreement – which is mainly the interpretation of quantitative results – led us to a better agreement. This is why we can claim that there is strong evidence that V.2 yields more consistent results, both quantitative and qualitative, when measuring ADL and IFX levels. On closer examination of this agreement, there are indicators which lead us to believe qualitative results are preferable in terms of reproducibility and therefore of greater potential use in clinical practice. Until now, there has been a lack of evidence on how different assays work (23, 32). A previous study, which was carried out on Crohn's disease samples, compared four techniques: RIA, ELISA, RGA (reporter gene assay) and EIA (enzyme immunoassay) for IFX levels and ADA determination. Good correlation between techniques was observed but discrepancies arose due to low limits of detection and sensitivity. A possible explanation is that the RIA technique is known to present increased 'sensibility' when compared to ELISA, with lower levels of drug interference. Furthermore, it was concluded that the monitoring of the individual patient should not be performed using different assays (33). In any case, these results are expected to be confirmed by large prospective studies comparing both methods in RA which aim to

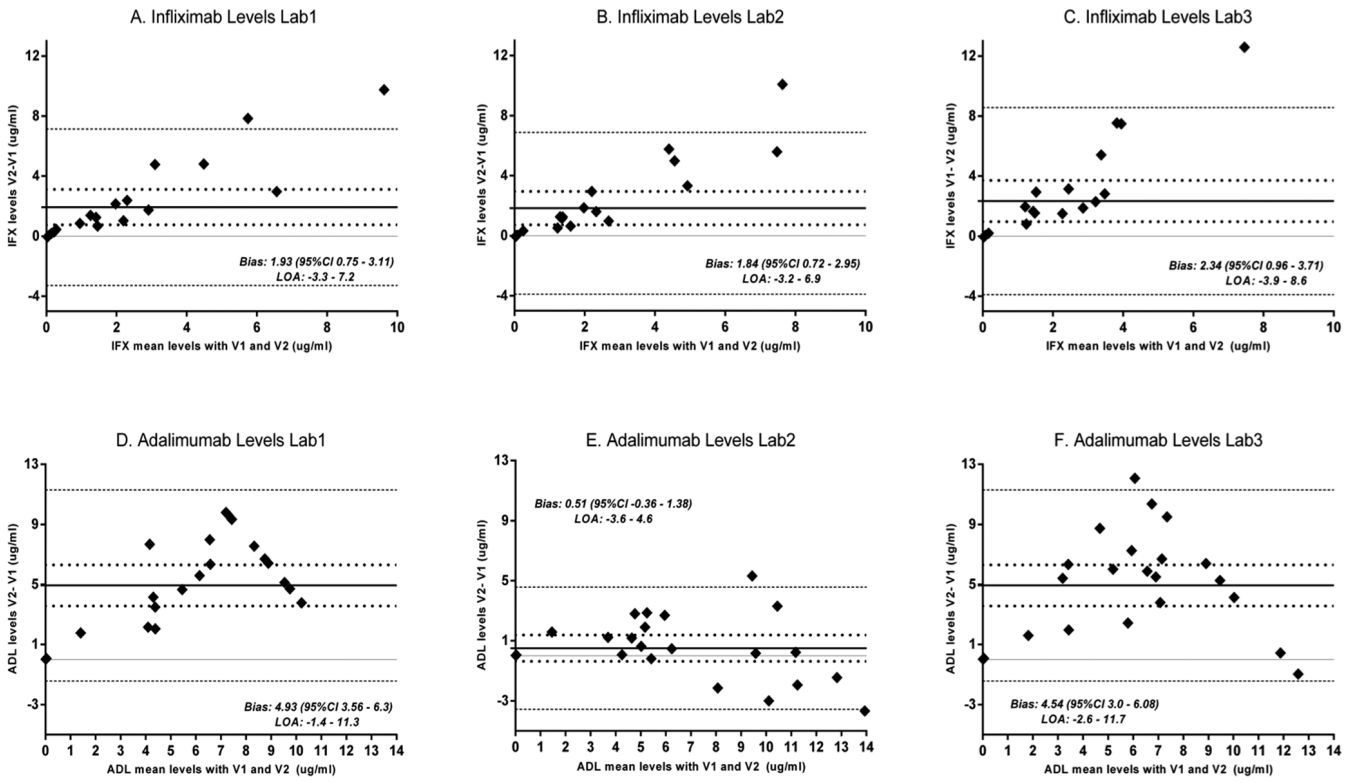

Fig. 2. A-F. Bland-Altman plots of differences between V.1 vs. V.2 for IFX and ADL in each laboratory.

develop a blueprint for standardisation. Currently, different ELISA assays are being utilised to measure IFX levels and IFX-ADA, some of which are academically developed, and others, commercial. Van de Casteele *et al*. found an acceptable interassay agreement using two in-house and one commercial ELISA (34).

One single-laboratory study in RA published in 2013 (35) compared two commercially available methods (Promonitor® kits and Sanquin Diagnostics) for the determination of anti-TNF drug levels which found a good linear association between both methods but with systematic overestimation in one and underestimation in the other one for ADL and IFX levels. Discrepancies described to date might be explained by the fact that IFX and ADL are well known complex protein molecules or that there is as yet no standardised model, or both. Sample manipulation, assay control, reagent stability, processing, storage and the use of different calibration standards could compromise the specificity, sensitivity and reproducibility of the assays which in turn may cloud associations between drug therapy and clinical response. The majority of this research has been performed in the field of inflammatory bowel disease in an attempt to associate these measurements with clinical outcomes (17, 33, 36, 37).

This inter-laboratory and inter-assay study attempted to address the technical discrepancies by comparing the results of three laboratories. Some of the most important differences were: a) standards and reagents were pre-diluted and b) variations in the manufacturer's cut-off points in both versions. Likewise, when comparing V.2 to V1, V.2 has a substantially reduced number of error-prone steps because most of the reagents are ready for use, decreasing the test procedure by more than half of total time (2.5h vs. 6h).

We present here the results of a comparative evaluation, as a first step toward consensus in setting up inter-laboratory reproducibility. To date unfortunately, there are neither gold standards nor guidelines available to monitor these drugs, for these reasons further multicenter studies will be needed as a prerequisite, in order to establish their use and to monitor treatment in clinical practice (38). The limitations of our study included: the small sample size, the impact of the results which are restricted in terms of location owing to kit availability and the fact that we have not used healthy control serum and known IFX or ADL spiked samples.

Qualitative measurements in V.2 produced better agreement which may be important when developing international standards to achieve uniform measurement in terms of developing guidelines in personalised patient management. Further inter-laboratory and inter-assay studies are required in order to standardise methods to monitor biological drugs and establish therapeutic reference ranges for each disease.

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