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ORIGINAL ARTICLE

Clinically important neutralizing anti-drug antibodies detected with an in-house competitive ELISA

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Abstract

Therapeutic drug monitoring of TNF-alpha inhibitors is crucial for evaluating patients with inflammatory diseases on a personalized level. It has been clinically observed that many patients receiving TNF-alpha inhibitors, with negative drug and anti-drug antibody results from bridging ELISA (bELISA), lose their drug response over time, despite dose optimization. Our aims were to develop innovative in-house competitive ELISAs (cELISAs) for the detection of neutralizing antibodies against infliximab and adalimumab and compare their results to reporter gene assay (RGA) and in-house bELISA. Furthermore, we aimed to evaluate patient anti-drug antibody results in regard to their clinical records and potential benefits of therapeutic drug monitoring with the novel cELISAs. Sera of patients treated with infliximab ($n = 46$) or adalimumab ($n = 31$), having undetectable drug levels, were tested with our in-house cELISA. Briefly, samples were incubated with a fixed amount of drug and the neutralizing capacity of the samples was determined. The cELISA results were compared to RGA and bELISA results using Spearman's correlation coefficient. Additionally, patient clinical data were evaluated in line with the results of cELISA, bELISA, and RGA using the Kaplan-Meier analysis and the Log Rank test. Both anti-infliximab and anti-adalimumab cELISAs showed very good correlation to RGA ($r = 0.932$, $p < 0.0001$ and $r = 0.947$, $p < 0.0001$, respectively). Furthermore, a positive result in anti-infliximab cELISA can predict treatment failure in 100% of patients with negative bELISA, while a positive result in anti-adalimumab cELISA can predict treatment failure in 80% of patients with negative bELISA. Taken together, we developed innovative cELISAs enabling quantification of functional and neutralizing anti-drug antibodies, comparable to RGA. The association between cELISA results and loss of drug response in patients identified clinically important anti-drug antibodies, as measured by cELISA.

Keywords Anti-adalimumab antibodies · Anti-infliximab antibodies · Competitive ELISA · Reporter gene assay

Rheumatology in Slovenia: Clinical practice and translational research

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Introduction

The treatment of inflammatory diseases, among them, Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, has changed dramatically after the development of tumor necrosis factor alpha (TNF-α) inhibitors as a result of TNF-α's role in disease pathogenesis [1–4]. Evidence has shown that TNF-α inhibitors are highly effective in inducing and maintaining clinical remission, showing significant improvements of symptoms and quality of patient life [5, 6]. However, a large proportion of patients does not respond to the induction therapy (primary non-response) or fails to respond and develops symptoms of relapse over time (secondary loss of response) [7]. The major reason for secondary failure is immunogenicity, which is a risk associated with all genetically engineered

proteins. The most frequently used TNF-α inhibitors, infliximab (IFX) and adalimumab (ADL), are foreign proteins to the patients' immune system. ADL is a full human monoclonal IgG antibody composed of human heavy and light chains, while IFX is a mouse-chimeric monoclonal antibody composed of the human constant and murine variable regions, both targeting biologic activity of TNF-α. They are both repeatedly administrated and can activate immunogenic responses and induce the formation of neutralizing and non-neutralizing anti-drug antibodies (ADA). ADA bind the drug and form immune complexes, which are removed from the blood; however, ADA also prevent the drug acting on TNF-α. The results of ADA formation are reduction of efficacy of the drug and appearance of adverse effects, such as infections and malignancies [6, 7]. However, the risk of serious infections and solid malignancies is low, so the benefits still outweigh the possible risks of serious adverse effects [8].

Therapeutic drug monitoring (TDM) of IFX and ADL is used to guide treatment in patients on TNF-α inhibitors. TDM has been increasingly used in routine practice in treating inflammatory bowel diseases, psoriasis, rheumatoid arthritis, and spondyloarthritis [9–12]. However, studies have shown that treatment optimization based on IFX, ADL, and ADA concentrations, using the clinically validated algorithm, is more effective than an empirical approach. Moreover, using TDM significantly reduces the cost of therapy per patient since treatment using IFX or ADL is very expensive. Additionally, when treating inflammatory bowel diseases, there are limited drug choices, meaning TDM has emerged as a strategy to optimize treatment and maximize benefits from these drugs, and may also improve adherence [13, 14]. TDM, testing for drug and possible ADA formation, is appropriate in four scenarios: after induction in primary non-responders and in secondary non-responders, during maintenance therapy and whenever restarting a drug, after the drug has not been used for some time [15]. In TDM, the time of sampling is very important because the drug concentrations change during the dosing interval. The most stable point is the drug trough concentration, where we can observe the lowest serum concentrations before the next application [16] and consequently, this time point is used in laboratories worldwide. The concentrations of drugs and also ADA are influenced by factors related to patients, treatment, and also to the utilized assays. The adequate serum concentrations of the drug are associated with sustainable clinical response in patients [17].

The most often used assays in routine practice are binding-based assays (bridging ELISA—bELISA and homogeneous mobility shift assay—HMSA) and the functional cell-based assay reporter gene assay (RGA). bELISA and HMSA cannot differentiate between neutralizing and non-neutralizing ADA, while the information about neutralization properties of ADA is important in studying/explaining loss of drug response. Therefore, RGA is the only method for detection of functional and neutralizing ADA, but it is also both costly and labor-intensive. Furthermore, all these methodologies are affected by different levels of the drug already present in patient sera, which also means that they only detect free ADA and not ADA in immune complexes [18–21].

For all the above reasons, alternative assays are warranted to obviate these limitations. Until now, some studies have utilized competitive-based assays [22, 23], using competitive ligand-binding methodologies for detection of neutralizing antibodies. The assay combines a simple protocol, low price, shorter analysis time, and, lastly and most importantly, detection of only neutralizing antibodies. However, there is no study published to date about the comparability with RGA.

The aim of this study was first, to validate a novel in-house competitive ELISA (cELISA) for the detection of neutralizing anti-IFX and anti-ADL antibodies and to compare the results with RGA and in-house bELISA. Secondly, patient results will be evaluated in regard to their clinical records and potential benefits of TDM performed with the new cELISAs will be presented.

Materials and methods

Patients

This study comprised of 46 patients' sera with IFX concentrations below 0.50 μg/mL and 31 patients' sera with ADL concentrations below 0.50 μg/mL. After patients signed an informed consent, their samples were collected immediately before infusion of a new IFX or ADL dose in the Departments of Gastroenterology and Rheumatology, at the University Medical Centre Ljubljana between August 2016 and December 2017. Patients' characteristics are provided in Table 1.

We included naïve samples from patients with inflammatory bowel or chronic rheumatic diseases, naïve samples from patients with positive rheumatoid factor, and samples of healthy blood donors to test matrix effect in cELISA.

This study was conducted as part of the National Research Program entitled "Systemic Autoimmune Diseases" (#P3-0314) and has been approved by the National Medical Ethics Committee (#99/04/15 and #38/01/16).

IFX and ADL detection

All samples included in the study were tested in the first step for IFX and ADL with in-house IFX or ADL ELISA. Briefly, TNF-α (PeproTech, NJ, USA) was coated onto plates overnight at 4 °C. The coated plates were washed, blocked, and incubated with samples, quality controls, and standard dose-response curve (Remsima™, Celltrion Healthcare, Incheon, Korea in IFX ELISA and Humira®, AbbVie, North Chicago, USA in ADL ELISA). Anti-IFX monoclonal

Table 1 Patients' characteristics

Characteristics	Anti-IFX group ($n = 46$)	Anti-ADL group ($n = 31$)
Sex		
Female, *n* (%)	23 (50)	18 (58)
Male, *n* (%)	23 (50)	13 (42)
Diseases		
Inflammatory bowel diseases, *n* (%)	22 (48)	19 (61)
Chronic rheumatic diseases, *n* (%)	24 (52)	12 (39)
Median age at sampling time, years (range)	52 (20–86)	46 (19–67)
Median time of IFX/ADL therapy, months (range)	12 (1–177)	24 (4–162)
Concomitant immunosuppressives, *n*		
Methotrexate	10	2
Methylprednisolone	10	–
Leflunomide	3	4
Azathioprine	3	1
Sulfasalazine	–	1
Budesonide	–	1
Mesalazine	2	–
No	18	22

antibody MA-IFX6B7 and anti-ADL monoclonal antibody MA-ADL40D8, both conjugated with horseradish peroxidase (HRP) (apDia, Turnhout, Belgium, KU Leuven, Belgium) [24, 25] were used to detect IFX and ADL binding, respectively. The enzyme reaction with TMB substrate (Thermo Fisher Scientific, MA, USA) was stopped using 0.18 M H_2SO_4 and absorbance measured at 450 nm (reference filter 680 nm) by a spectrometer (Tecan, Mannedorf, Switzerland). Results were calculated based on the standard dose-response curve using IFX (Remsima™) or ADL (Humira®) at concentrations ranging from 5 to 120 ng/mL. In-house IFX and ADL ELISA were developed and validated according to CLSI guidelines [26]. All samples were also tested for IFX and ADL levels with RGA (IFX and ADL RGA) according to manufacturer's instructions (Euro Diagnostica, Malmo, Sweden).

Anti-IFX and anti-ADL detection

Competitive ELISA

TNF-α (PeproTech, NJ, USA) diluted in phosphate-buffered saline (PBS) was coated onto Nunc MaxiSorb microtiter plates (Thermo Fisher Scientific, MA, USA) overnight at 4 °C. The coated plates were washed with PBS containing 0.05% (*v*/*v*) Tween-20 (Sigma Aldrich, MO, USA) and blocked with 1% bovine serum albumin (BSA)/PBS for 2 h at room temperature (RT). Samples were diluted from 1:20 to 1:2560 and pre-incubated with a fixed amount of IFX or ADL (40 ng/ml) linked with HRP for 30 min at 37 °C and then added to the TNF-α plate for 1 h at RT. The reactions were detected using TMB substrate and the results were calculated according to the threshold defined, using samples which were negative in anti-IFX and anti-ADL RGA. The principle of methodology is graphically shown in Fig. 1, as compared to anti-IFX and anti-ADL RGA. Neutralizing ADA bound the added drug in the assay and prevented the drug binding to the plate with TNF-α. As a result, a decrease in signal was detected, while in the sample negative for neutralizing ADA, the drug was free and could bind to the TNF-α on the plate and the signal was high. In cELISA, the standard curve comprises of HRP-linked IFX/ADL between 40 and 5 ng/mL. Imprecision of the cELISA was evaluated by testing two positive samples five times in one assay. The experiment was repeated five times [26]. Samples with anti-ADL and anti-IFX were tested for specificity in "opposite" cELISA. Matrix effect and interference of rheumatoid factors were also investigated.

Reporter gene assay

RGA (anti-IFX and anti-ADL RGA) was performed following manufacturer's instructions (Euro Diagnostica, Malmo, Sweden) using iLite TNF-alpha Assay Ready Cells. The threshold was defined testing 30 biological-naïve patient sera. Calculations were done according to the threshold defined in verification. Methodology principle is graphically shown in Fig. 1.

In-house bridging ELISA

Briefly, IFX (Remsima™) or ADL (Humira®) diluted in PBS were coated overnight at 4 °C to microtiter plates. The coated plates were washed, blocked, and incubated with samples, quality controls, and standard dose-response curve. IFX (Remsima™) and ADL (Humira®)-linked with HRP were used to detect anti-IFX or anti-ADL binding, followed by TMB substrate and stop solution 0.18 M H_2SO_4.. Standard dose-response curve was prepared with varying concentrations of highly specific monoclonal anti-IFX MA-IFX10F9 or anti-ADL MA-ADL6A10 (apDia, Turnhout, Belgium, KU Leuven, Belgium) [27, 28] in a range from 5 to 0.2 ng/mL. IFX and ADL were conjugated with HRP using Lynx conjugation kit according to manufacturers' instructions (BioRad, Hercules, USA). In-house anti-IFX and anti-ADL bELISA were developed and validated according to CLSI guidelines [26].

Statistical analysis of correlations between methods

Correlations between cELISA and bELISA/RGA were calculated using Spearman's correlation analysis. An overall agreement (Kappa coefficient) was estimated according to negative

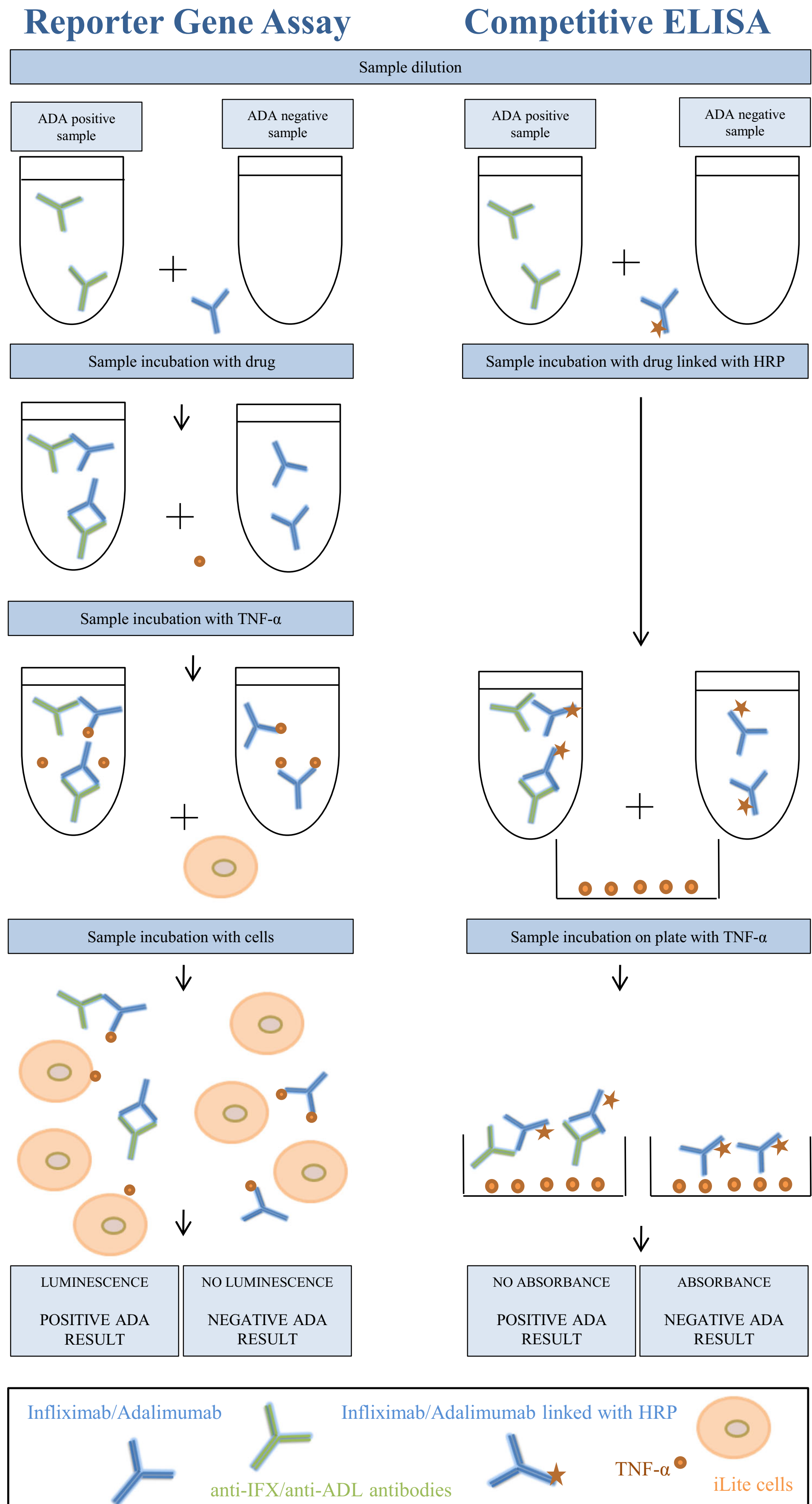


Fig. 1 Methodologies of RGA and cELISA for ADA detection (legend: ADA anti-drug antibodies)

and positive results. The Kaplan-Meier analysis and Log Rank test were used for statistical analysis of the patients' clinical data link to cELISA results. The statistical software package SPSS version 22 (IBM, NY, USA), GraphPad version 6 (GraphPad software, CA, USA) and Microsoft Excel 2010 were used for analyses. P values < 0.05 were considered significant.

Results

IFX and ADL detection

All samples included in the study were analyzed for IFX or ADL concentrations with in-house IFX or ADL ELISA and also confirmed in IFX or ADL RGA.

Anti-IFX and anti-ADL detection

cELISA

Repeatability or within-run imprecision for anti-IFX and anti-ADL cELISAs was below 12%, between-run imprecision was below 18%, and within-laboratory imprecision was below 20%.

For threshold determination in anti-IFX cELISA, 21 samples negative in anti-IFX RGA were used, while in anti-ADL cELISA, 23 samples negative in anti-ADL RGA were used. Because of a non-linear response between drug concentrations and OD responses in cELISA, the values for samples were calculated according to the curve and expressed in ng/mL. We calculated the average OD responses from samples and the standard deviation between results and determined the threshold at 99.5% confidence interval. In anti-IFX cELISA, the threshold was 23 ng/mL (which represents 43% inhibition of added Remsima™), while in anti-ADL cELISA, the threshold was 18 ng/mL (which represents 55% inhibition of added Humira®).

For each assay, we calculated the OD of the defined threshold (23 ng/mL in anti-IFX cELISA and 18 ng/mL in anti-ADL cELISA) from the standard curve. According to calculated OD, the corresponding titer was calculated in the samples. Samples were diluted from 1:20 to 1:2560 and using OD threshold and the sample dilutions curve, we calculated the titer, where the samples dilution curve crossed the threshold. The titer was calculated as juncture from the curve and the threshold.

The assays are specific because all samples containing high anti-IFX/anti-ADL concentrations were negative in opposite cELISA. Samples of healthy blood donors, samples from naïve patients, and naïve samples, with high levels of rheumatoid factor, all had negative responses showing no matrix effect and no interference of rheumatoid factor.

Correlations between cELISA and RGA/bELISA

Samples with negative levels of anti-IFX/anti-ADL antibodies (titer < 20 in RGA and cELISA) and very high levels of anti-IFX/anti-ADL (titer > 2560 in RGA and cELISA) were omitted from further calculations due to non-defined exact values.

For both anti-IFX and anti-ADL titers, we found very strong correlations between measurements in cELISA and RGA. Spearman's correlation coefficient for anti-IFX was $r = 0.932$ ($p < 0.0001$) and an overall agreement between negative and positive results was 100% resulting in kappa coefficient 1.000. Spearman's correlation coefficient for anti-ADL titers was $r = 0.947$ ($p < 0.0001$) and overall agreement between positive and negative results was 100% (kappa coefficient 1.000) (Fig. 2, Table 2).

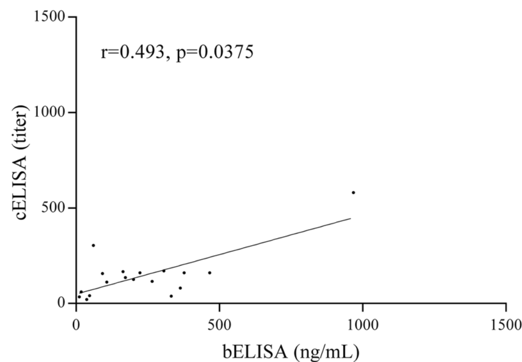
cELISA results of anti-IFX antibodies showed moderate correlation with bELISA results ($r = 0.493$, $p = 0.0375$), while cELISA results of anti-ADL antibodies showed very strong correlation to bELISA results ($r = 0.952$, $p = 0.0001$). Agreement between bELISA and cELISA was 78% for anti-IFX and 81% for anti-ADL samples between negative and positive results. Kappa coefficient between anti-IFX bELISA and cELISA was 0.547 and between anti-ADL bELISA and cELISA 0.627 (Fig. 2, Table 2).

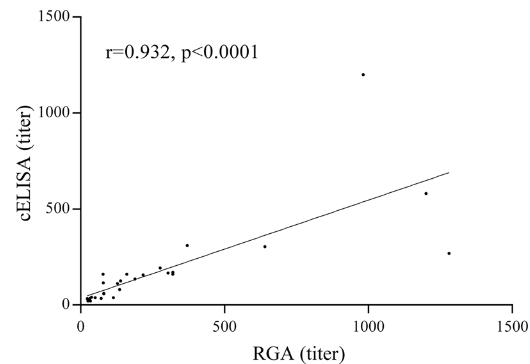
Clinical validation of cELISA

In clinical validation of cELISA, we included samples that had negative anti-IFX or anti-ADL levels using bELISA at sampling time. The negative samples from bELISA were all subsequently tested by cELISA. Using the Kaplan-Meier survival analysis, we only included samples of patients who continued with the therapy, meaning they had received at least one more application of the drug after sampling time. We compared samples that were negative in both assays, in bELISA and cELISA, with the samples negative in bELISA and positive in cELISA according to clinical status on follow-up (observation time). Flow charts are presented for the selected anti-IFX group of patients (Fig. 3a) and the anti-ADL group of patients (Fig. 3b) and their clinical validation.

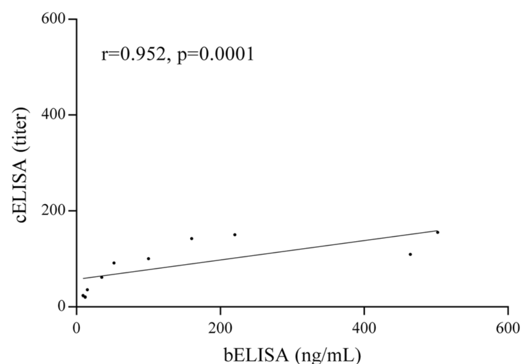
The therapy continued for 4/7 anti-IFX positive samples, 10/13 anti-IFX negative samples, 5/6 anti-ADL positive patients, and 11/13 negative patients in cELISA. From further studies in anti-IFX group, we observed 100% loss of response in patients with negative bELISA and positive cELISA, while 50% loss of response in patients with both ELISAs negative. In the anti-ADL group, 80% loss of response was observed in patients with negative bELISA and positive cELISA, while 45% loss of response in patients with both ELISAs negative. According to that, we can conclude that a positive result in cELISA can predict treatment failure in patients treated either with IFX or ADL.

a anti-IFX assays





b anti-ADL assays



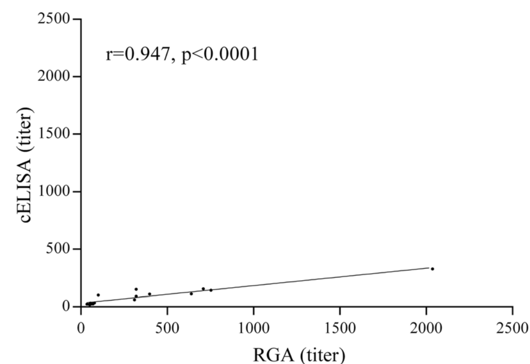


Fig. 2 Correlations of cELISA results of **a** anti-IFX and **b** anti-ADL with bELISA or RGA

The Kaplan-Meier analysis and Log Rank test of these data showed no statistically significant differences in anti-IFX group between the group of samples negative in both assays and the group of samples negative in bELISA, but positive in cELISA ($p = 0.863$). Both groups of patients experienced a similar time period before treatment failure.

On the other hand, in the anti-ADL group, the Kaplan-Meier analysis and Log Rank test of these data showed statistically significant differences between the group of samples negative in both assays and the group of samples negative in bELISA, but positive in cELISA ($p = 0.024$). Patients with positive cELISA experienced a shorter time period before treatment failure compared to patients negative in both assays.

Discussion

The results showed that TDM implementation to screen patients for drug concentrations and additionally, levels of ADA, can improve treatment optimization. The recommendations, recently presented for TDM in patients with inflammatory

Table 2 Comparison between results from cELISA, RGA, and bELISA

		RGA				bELISA			
		Anti-IFX		Anti-ADL		Anti-IFX		Anti-ADL	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
cELISA	Negative	16	0	13	0	13	3	13	0
	Positive	0	30	0	18	7	23	6	12
Number of samples		46		31		46		31	
Agreement (%)		100		100		78		81	
Cohen's kappa coefficient (95% CI)		1.000		1.000		0.547 (0.299–0.795)		0.627 (0.358–0.895)	

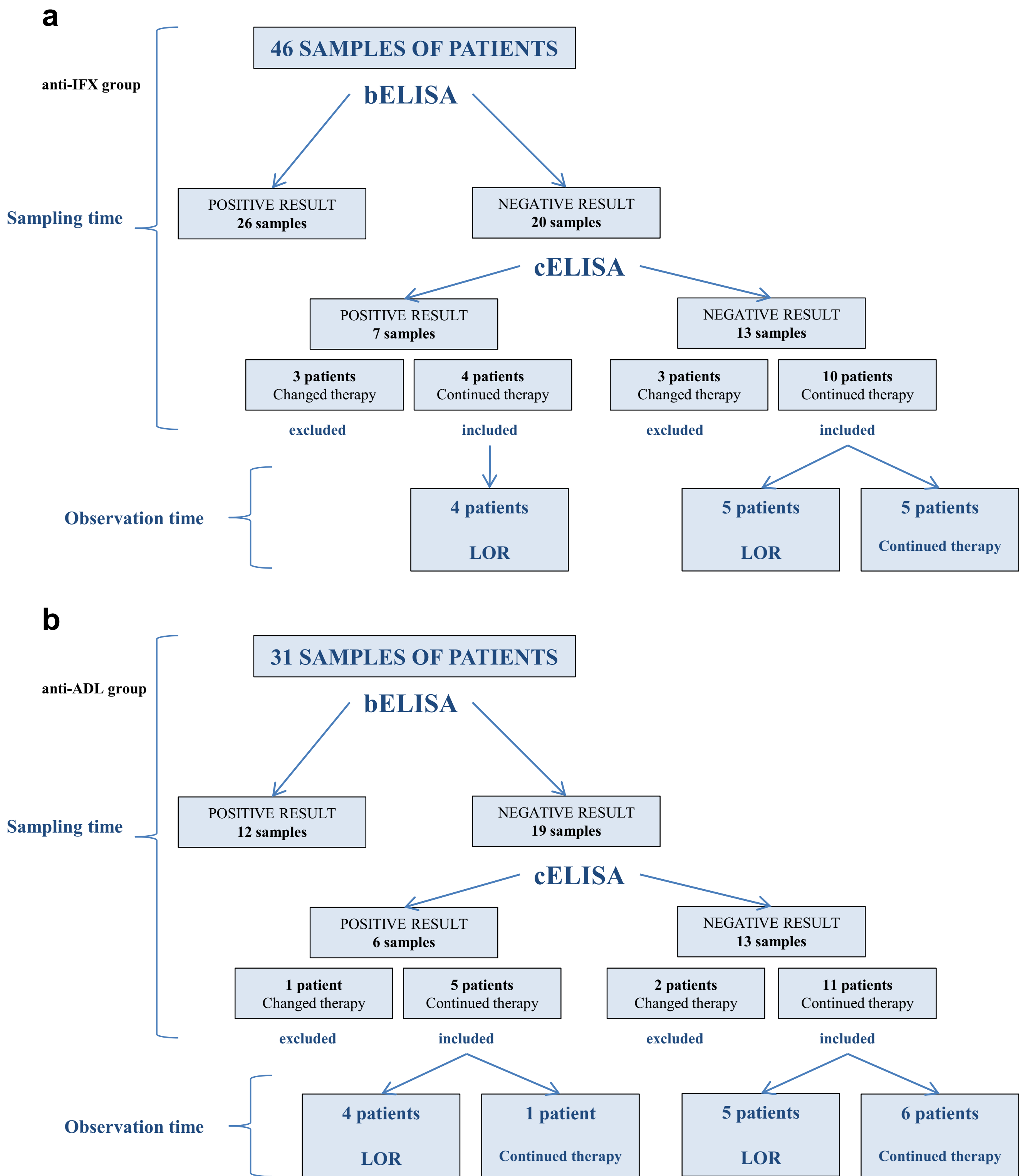


Fig. 3 Flow chart of cELISA clinical validation (legend: LOR loss of response)

bowel diseases, can help guide clinicians to optimize treatment [15]. The clinical problem is that it is impossible to differentiate between drug failure due to ADA or due to non-immunogenic pharmacokinetic mechanisms, including reduced bioavailability of the drug, high drug consumption due to an active disease, and poor adherence to application,

among others. Specifically, recent studies reported the overall adherence to self-administered TNF-α inhibitors was low. Therefore, measuring drug serum concentration and neutralizing ADA to distinguish patients at highest risk of non-adherence may improve the safe, effective, and efficient use of TNF-α inhibitors [29].

Many different methodologies have been utilized; however, all have their advantages and disadvantages [19, 30]. Thus, laboratories need to carefully choose the appropriate methodology, especially for ADA detection. bELISA is most commonly used, but clinicians have observed that a great proportion of patients with negative results in bELISA develop loss of response, despite dose optimization.

In our study, an innovative cELISA was developed for anti-IFX and anti-ADL detection. The assay protocol was adjusted from IFX and ADL ELISAs with the same TNF-α concentration and blocking reagents. The detection system was changed to reduce steps and time for analysis; therefore, we used labeled drug, which was spiked in tested samples. After sample incubation, we performed the detection step with substrate solution. The protocol is short and user- and finance-friendly for routine work in laboratories performing many analyses per day.

For threshold determination, the samples which were negative in RGA were tested to validate the assay comparable to the functional RGA, which detects only neutralizing, clinically important ADA. We calculated the threshold using the 99.5% confidence interval. The threshold represents 43% inhibition in anti-IFX and 55% inhibition in anti-ADL cELISA. These thresholds are comparable with the thresholds used in other studies, using cELISA. One study calculated EC50 and also percentage of inhibition [22], while in another, they reported a 50% inhibition [23]. However, our protocol uses dilutions between 1:20 to 1:2560 and the calculations used in RGA and thus defines the actual titer of ADA, similarly to RGA.

Our cELISAs showed good imprecisions calculated from low positive and high positive samples. All imprecisions met validation criteria with coefficient of variation below 20%. There was no matrix effect, as defined by testing naïve sera samples. Our assays were specific, which was shown by testing anti-IFX positive samples in anti-ADL cELISA and vice versa.

The current report is the first to our knowledge to compare results of cELISA with RGA and show comparable results of cELISA in identifying functional and neutralizing ADA against the drug. In our cELISA and RGA, the same ratio between drug and sample dilution was used, with a dilution 1:20 and IFX/ADL concentration of 40 ng/mL. All additional dilutions were the same between the two methods (from 1:20 to 1:2560) yielding similar results between cELISA and RGA.

Until now, one study compared results of cELISA with bELISA using a low number of samples [22]. Namely, from the group of patients on IFX therapy, they found seven samples of patients with low IFX concentrations and these seven samples were further analyzed for anti-IFX antibodies using cELISA and bELISA. Three samples showed low inhibitory activity, while four showed larger inhibitory activity in cELISA. However, by using bELISA, ADA were not detected in these samples [22].

In our study, the correlation between cELISA and bELISA was good, with even better correlation observed between cELISA and RGA. This is understandable, since the methodologies between cELISA and RGA are more comparable than bELISA and cELISA.

bELISA is widely used in laboratories due to its simplicity and quick protocol; however, the assay has some limitations. bELISA cannot detect IgG4 antibodies due to their bispecificity and cannot distinguish between neutralizing and non-neutralizing ADA. Another problem is the possibility of false positive results due to the presence of high levels of rheumatoid factor in patients with rheumatoid arthritis. cELISA solves these issues, since it can detect both IgG4 antibodies and neutralizing antibodies. Also, high levels of rheumatoid factor do not interfere in our cELISA assay. We can conclude that our in vitro cELISA mimics the neutralizing competitive binding of the in vivo RGA. The output result in bELISA is ADA concentration (due to monoclonal antibodies used to prepare the standard curve), while cELISA and RGA use titers as the output result, which is preferential due to the high difference between affinities of ADA. cELISA and RGA detect neutralizing ADA, but do not detect non-neutralizing antibodies which affect the pharmacokinetics and increase the drug clearance. The question arises about the importance of non-neutralizing antibodies, in view of the dose and interval optimization, to maintain the patient in remission, as well as the importance of positive ADA levels in samples with high drug concentrations. The majority of assays are able to detect ADA only in samples with low drug concentrations. In our case, we tested samples with concentrations below 0.50 μg/ml of IFX or ADL in cELISA, bELISA, and RGA.

The research of new assays is currently focusing on drug-tolerant assays, meaning the assay can detect ADA in samples with positive drug levels. However, the published data on drug-tolerant assays showed no clinical relevance of ADA detected in the presence of the drug [31].

In the last part, we presented the clinical validation of cELISA. According to our results, the positive cELISA can predict treatment failure in patients on therapy with either IFX or ADL. Moreover, the Kaplan-Meier analysis showed that patients negative in bELISA and positive in cELISA had a shorter time to treatment failure compared to patients negative in both assays in anti-ADL group. While in the anti-IFX group, both groups exhibited a similar time to failure. But as stated before, the descriptive statistics showed the importance of positive cELISA in samples with negative bELISA.

Clinician decisions could be different if they had more timely data on ADA available. This would not only prevent expensive therapy which is not likely to work, but also could

enable patients to receive new therapy prior to worsening of symptoms.

To conclude, we developed and validated a novel cELISA, which detects functional and neutralizing antibodies in patients taking IFX or ADL. RGA is the only method that detects neutralizing antibodies to date and represents the assay in which the threshold was clinically confirmed. The correlations between cELISA and RGA for neutralizing anti-IFX and anti-ADL detection were calculated showing comparable results to RGA. The clinical data on patients show the usefulness of cELISA results in samples negative in bELISA. Thus, cELISA demonstrates greater clinical utility than bELISA alone, as well as represents an assay that is cost-, time-, and labor-effective.

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Author contributions All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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Compliance with ethical standards

Disclosures None.

Ethical approval This study was conducted as part of the National Research Program (#P3-0314) and has been approved by the National Medical Ethics Committee (#99/04/15 and #38/01/16). It has therefore been performed in accordance with the ethical standards laid down in 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all individuals included in this study.

Disclaimer No funding commercial organizations played a role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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