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## Accurate and rapid microfluidic ELISA to monitor Infiximab titers in patients with inflammatory bowel diseases

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Inflammatory bowel disease (IBD) is a term used to describe disorders that involve chronic inflammation in the gastrointestinal tract, affecting more than 6.8 million people worldwide. Biological therapy is used in the most severe cases of IBD where anti-tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) antibodies are the first choice for a biological treatment. When administered to patients, these antibodies interact with TNF- $\alpha$ , usually overexpressed in these diseases, neutralizing its biological activity. Because of the chronic nature of these diseases, a recurring administration of the therapeutic antibodies is required, thus making therapy monitorization essential for the correct management of these diseases. The aim of this work is the development of an enzyme-linked immunosorbent assay (ELISA) microfluidic biosensor to quantify the therapeutic antibodies in IBD patient plasma samples, where the commercial monoclonal antibody Infiximab (IFX) is used as a model target. By providing a faster and more accurate measurement of IFX, the proposed method leads to improved therapy scheduling and a reduced risk of endogenous anti-drug antibodies (ADAs) reducing the efficacy of the treatment. The time needed between sample insertion and result output for the microfluidic ELISA (mELISA) is 24 minutes, drastically shorter than the time required by the conventional ELISA (cELISA). The mELISA presented in this work has a LoD of  $0.026 \mu\text{g mL}^{-1}$ , while commercially available solutions provide a LoD of  $0.15 \mu\text{g mL}^{-1}$ . Results acquired by the mELISA are highly correlated with the results obtained from the cELISA ( $r = 0.998$ ;  $R^2 = 0.996$ ;  $p < 0.0001$ ), demonstrating the validity of the microfluidic approach for the quantification of IFX from patient plasma and its potential for use at the point-of-care (POC).

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## Introduction

Anti-TNF- $\alpha$  antibodies are one of the most profitable therapeutic proteins.<sup>1</sup> In 2018, IFX and Adalimumab (ADL) together have generated a profit of USD 25.8 billion (€22 billion), representing of 41% of the total profit generated by monoclonal antibodies.<sup>1,2</sup> IFX is a chimeric human-murine

IgG1 monoclonal antibody approved for the treatment of chronic inflammatory diseases such as Crohn's disease (CD), ulcerative colitis (UC), ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis and chronic plaque psoriasis, where TNF- $\alpha$ , a pro-inflammatory cytokine, is overexpressed.<sup>3,4</sup> Due to the chronic nature of these diseases, a recurring administration of the treatment is necessary to minimize the symptoms and the progression of the disease.<sup>5</sup> However, only the more severe forms of these diseases are treated with therapeutic antibodies, due to the high cost associated with the treatment and subsequent burden on public health systems. As an example, the costs for the healthcare system in Portugal for a single vial (100 mg) of these antibodies is € 461.43.<sup>6</sup> The recommended therapeutic dose for the administration of IFX is 5 to 10 mg  $\text{kg}^{-1}$  on week 0, 2, 6, followed by periodic administrations every 4 weeks in active form of CD or UC. Considering a person of average weight (70 kg) the treatment costs for the IFX alone ranges between € 27k to € 47k in the first year of treatment.<sup>4–7</sup>

ADAs are produced in patients who are under antibody therapy and are an important biological immune response

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that leads to treatment failure.<sup>8</sup> ADAs interact with therapeutic antibodies forming immunocomplexes, increasing the drug clearance in circulation which results in ineffective drug dosages.<sup>9</sup> This undesired response can be partially addressed by a dose escalation of the therapeutic antibody, using shorter intervals between administrations, switching to another anti-TNF- $\alpha$  antibody, or adding a concomitant immunomodulator therapy to maintain a good clinical response.<sup>10–14</sup> To achieve a correct therapy management, it is essential to monitor both the therapeutic antibody and the ADAs levels in the patients' blood.<sup>8,15</sup> Most authors recommend a therapeutic range for IFX concentration between 3 to 7  $\mu\text{g mL}^{-1}$ , while considering values until 10  $\mu\text{g mL}^{-1}$  acceptable. IFX levels lower than 3  $\mu\text{g mL}^{-1}$  are highly correlated with the development of ADAs.<sup>7,16–18</sup> The standard measurement of IFX and ADAs levels in patient's plasma is usually performed by ELISA, which provides adequate sensitivity and reproducibility.<sup>8,15,19</sup> However, a cELISA does not provide the physician with a result for IFX quantification within the time frame of the medical appointment, making accurate therapeutic adjustments impossible. This in turn leads to a possible increase of undesired immune responses for the patient and consequent costs for the healthcare system. Therefore, there is a need to use a faster, but equally reliable method to monitor these therapies.

Microfluidic immunoassays have been under development by different research groups to answer needs in different fields ranging from medical diagnostics and monitoring of biopharmaceutical production to agriculture and food analysis.<sup>20–24</sup>

When compared to cELISA, the major advantage is a decrease in contamination possibilities, due the reduced number of handling steps, resulting in the reduction of false positive results. Other significant advantages include the use of small sample volumes, as well as low reagent and time consumption.<sup>20,21,25–27</sup> These devices are often developed in an user-friendly manner, leading to easy-to-use devices that do not require qualified technicians for operation. Microfluidic devices, in general, also allow for higher levels of integration and automation.<sup>20,22–26</sup> ELISA has been receiving attention for application in POC diagnostics, through the combination of new approaches in the detection methodologies and read-out equipment. Previous reports refer to the possibility of achieving detection sensitivities in the picomolar range for specific proteins.<sup>20–23</sup> Further development of more microfluidic bio-sensor systems will allow physicians to closely monitor patient health during the moment of consultation, leading to more accurate diagnostics. The portability aspect of mELISA devices supports their use in remote locations, where resources might be scarce, like isolated villages, soldiers in active combat, or astronauts in space, which is an added benefit of these types of systems.<sup>26</sup>

At the time of writing, there are 6 commercially available lateral flow assays to quantify IFX (or ADL) and the respective ADAs in patient's plasma or serum. These are sold as individual tests: Quantum Blue Infliximab, Anti-Infliximab, Adalimumab, and Anti-Adalimumab, from Bühlmann Laboratories, and RIDAQUICK IFX Monitoring, and ADM

Monitoring from R-Biopharm AG. However, the only products with authorization to be used as medical devices are Quantum Blue Infliximab and Quantum Blue Anti-Infliximab, where the license is restricted to Canada. The other POC devices have been used only for research purposes. All the previously mentioned assays use a lateral flow approach, and the assays' duration varies between 16 to 21 min.<sup>28–33</sup> The purpose of this study is to demonstrate that an ELISA based in microfluidics for monitorization of IFX is a choice for POC, which combines speed, sensitivity and easy to use.

## Experimental

The main steps for Infliximab quantification in patient samples followed in this work are illustrated in Fig. 1.

### Patient sample treatment

Patient plasma samples with inflammatory bowel disease treated with infliximab and subjected for therapeutic monitoring arrived in dry ice and were stored at  $-80\text{ }^{\circ}\text{C}$ . Patients' samples were processed by the University Hospital Southampton NHS Foundation Trust and the University of Southampton with the approval number Ibissuk65214. Informed consent was obtained for any experimentation with human subjects. Healthy blood samples used as negative controls were obtained from Instituto Português do Sangue e da Transplantação, IP (IPST, IP). To separate the plasma from the whole blood, blood was centrifuged at 16 900g for 5 minutes at  $4\text{ }^{\circ}\text{C}$ . The plasma was collected and diluted 10 and 100 times in serial dilutions in a solution of 1% (w/v) bovine serum albumin (BSA, A3733, Sigma-Aldrich, MO, USA) in 0.01 M phosphate-buffered saline at 0.5% (v/v) Tween 20 (PBS-T) and immediately stored at  $-80\text{ }^{\circ}\text{C}$ .

### Microfluidic structure fabrication

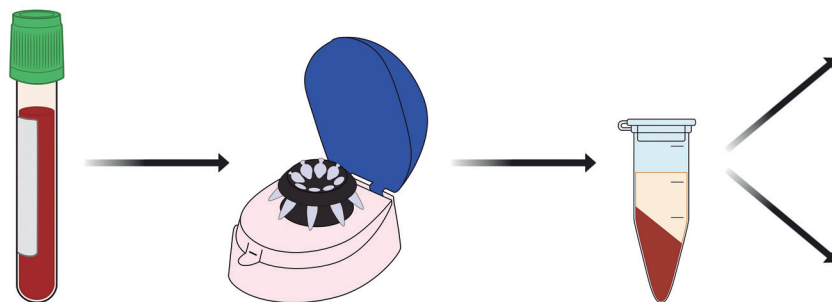
A microfluidic structure depicted in Fig. 2 was fabricated following standard microfabrication procedures previously described in literature.<sup>35</sup> Briefly, a 200 nm thick layer of aluminum was first deposited on the glass substrate through sputtering (Nordiko 7000, Nordiko Technical Services Ltd, Hampshire, UK). The aluminum layer was then covered with a 1.5  $\mu\text{m}$  thick layer of a positive photoresist (PFR 7790G, JSR, CA, USA). The coated sample was then patterned through direct write lithography ( $\lambda = 405\text{ nm}$ ; DWLii direct write laser photolithography system, Heidelberg Instruments, Heidelberg, Germany) according to a previously prepared CAD file. The exposed aluminium regions were removed by wet etching using a commercial aluminium etchant (Techni Etch Al80 Aluminium etchant; Microchemicals, Ulm, Germany) followed by removal of the photoresist with pure acetone. This aluminium hard mask was then used to fabricate an SU-8 mold. SU-8 2015 (Microchem, MA, USA) was spin-coated (Laurell Technologies Corp., PA, USA) on top of a clean silicon substrate (University Wafer, MA, USA). The photoresist was soft baked ( $95\text{ }^{\circ}\text{C}$ , 5 min), followed by exposure for 30 s to a 400 W

## A - Infliximab quantification in patients' plasmas

1. Blood collection in hospitals and transport to the laboratory

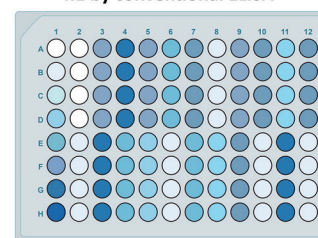
2. Blood centrifugation for 5 min at 16.900 xg

3. Plasma collection



4. Infliximab quantification

4.1 by conventional ELISA



4.2 by microfluidic ELISA



## B - Indirect ELISA for Infliximab quantification

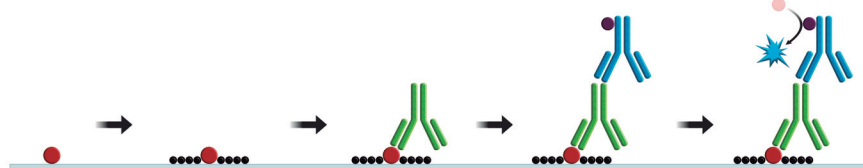
1. TNF- $\alpha$  coating

2. Blocking 3% BSA<sup>4.1</sup> or 1% Casein<sup>4.2</sup>

3. Plasmas patients' loading

4. Secondary antibody label with HRP

5. Substrate addition TMB<sup>4.1</sup> or Luminol<sup>4.2</sup>



5. Signal detection colorimetric<sup>4.1</sup> or chemiluminescence<sup>4.2</sup> measurement

**Fig. 1** Diagram illustrating the main steps for Infliximab quantification from the blood collection to plasma measurement by ELISA. (A) Experimental procedure performed to quantify infliximab in patient plasma samples, where the (A1) blood samples were collected in hospitals immediately before the dose administration, followed by sample transportation to the laboratory to be analysed. (A2) The blood is separated by centrifugation at 4 °C for 5 min at 16 900g to obtain the plasma, (A3) followed by aliquoting in microtubes and storage at –80 °C, including the dilution of 100 times used to quantify IFX. (A4) IFX was quantified (A4.1) by conventional ELISA, and (A4.2) by microfluidic ELISA. Depending on the approach used to quantify IFX the (A5) signal detection method differs in a colorimetric method in conventional ELISA using a microplate reader at  $\lambda = 450$  nm, or a chemiluminescence method in microfluidic ELISA using a microscope coupled with a digital camera. (B) Scheme of the indirect ELISA used for Infliximab quantification. (B1) TNF- $\alpha$  coating, (B2) blocking of the free surface with (A4.1) 3% BSA or (A4.2) 1% casein, (B3) calibration curve and plasma patients' loading, (B4) loading of secondary antibody label with HRP, and finally the (B5) addition of the substrate to be oxidized in the presence of HRP and hydrogen peroxide, generating a soluble blue product or chemiluminescence at approximately  $\lambda = 425$  nm. BSA – bovine serum albumin, ELISA – enzyme-linked immunosorbent assay, HRP – Horseradish peroxidase, IFX – Infliximab, TNF- $\alpha$  – tumour necrosis factor- $\alpha$ .

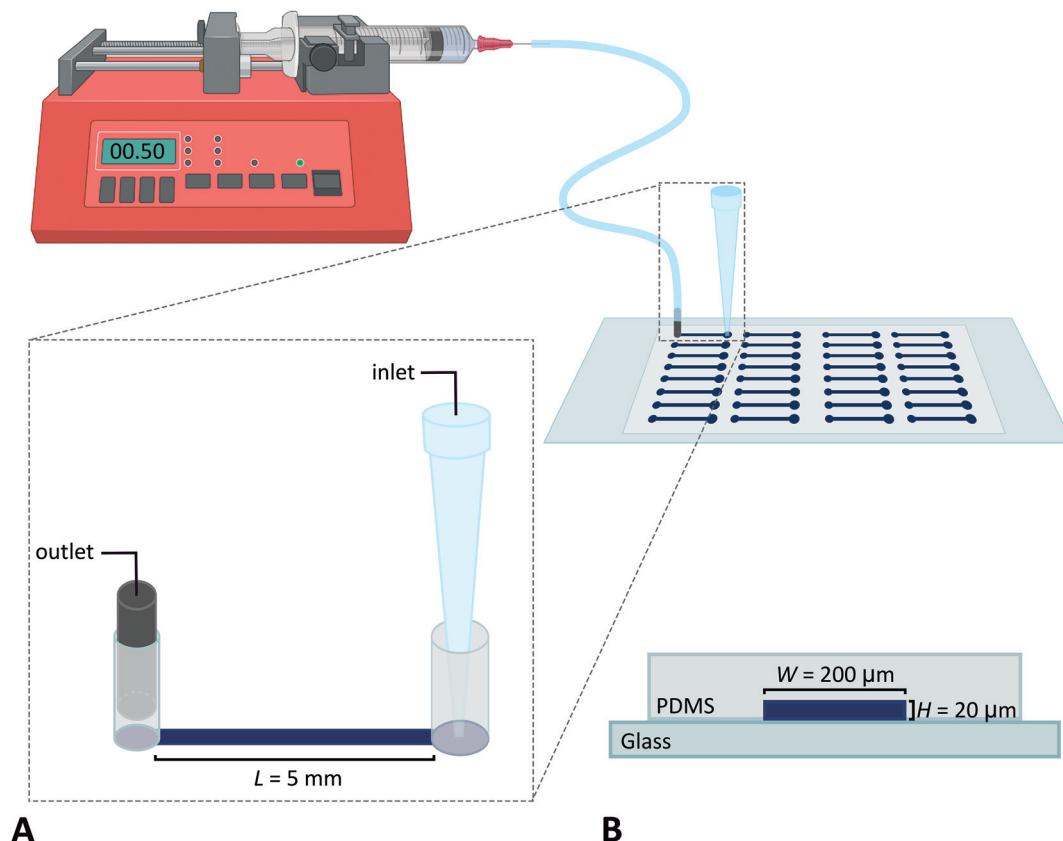
UV light (UV Light Technology Limited, Birmingham, UK) through the hard-mask, followed by a second baking step (95 °C, 5 min). After development of the non-exposed SU-8 in a propylene glycol monomethyl ether acetate (PGMEA) bath, the mold was baked for 15 min at 150°.

The SU-8 mold was then used to produce several polydimethylsiloxane (PDMS) microfluidic structures. To prepare the PDMS elastomer, a 10 : 1 weight ratio of PDMS pre-polymer to curing agent was mixed (Sylgard 184, Dow Corning, MI, USA), and degassed for 45 min. The mixture was poured into the Petri dish containing the SU-8 mold to a height of around 0.6 cm, followed by a curing step (90 min, 70 °C) in a convection oven (100–800, Oven loading, Schwabach, Germany). The cured PDMS was cut using a scalpel, and then peeled off the mold. Access ports for the PDMS structures were punched with blunt 18 and 20 ga needles (LS18 and LS20 K, Instech Laboratories, Inc., PA, USA) to create inlets and outlets,

respectively. PDMS structures were sealed against clean microscope glass slides (631-1550, VWR, PA, USA) by treating both surfaces using an oxygen plasma cleaner (PDC-002-CE, Harrick Plasma, NY, USA) at 800 mTorr oxygen pressure, at the medium power setting (11 W) for 5 min. The treated surfaces were brought immediately into contact, creating a permanent bond. The mELISA was performed after, at least, 24 hours post-bonding to allow a complete recovery of the PDMS hydrophobicity and avoid inconsistency in molecular physisorption.<sup>36</sup>

### Infliximab quantification by indirect microfluidic ELISA

The mELISA was performed at RT in straight microfluidic channels described previously (shown schematically in Fig. 5).<sup>35</sup> The liquids were flowed into the microchannels by applying negative pressure to the outlet using a 12-channel syringe pump (NE-1200, New Era Pump Systems, Inc., NY,



**Fig. 2** Schematic representation of mELISA. (A) Setup used for the mELISA, where (B) cross-section diagram of the microchannel are illustrated. ELISA – enzyme-linked immunosorbent assay, mELISA – microfluidic ELISA.

USA). To connect the pump to the PDMS device, 1 mL syringes (U-100, CODAN Medical ApS, Lensahn, Denmark) with 20 ga blunt needles, were connected to polyethylene tubing (BTPE-90, Instech Laboratories, Inc., PA, USA) fitted with metal adapters (SC 20/15, Instech Laboratories, Inc., PA, USA). All steps in the mELISA started with a flow rate of  $5.5 \mu\text{L min}^{-1}$  in order to overcome the initial hydraulic resistance of the channel. When all 12 channels had an active flow, requiring around 30 s to achieved, the flow rate was changed to  $0.5 \mu\text{L min}^{-1}$  and the time for the duration of that step started counting. The washes were performed at  $5.5 \mu\text{L min}^{-1}$  for 1 min. All reagents used in the assay were diluted in PBS.

Each channel was coated with  $50 \mu\text{g mL}^{-1}$  of TNF- $\alpha$  for 10 min. During the optimization process, lower TNF- $\alpha$  concentrations were tested, however they do not provide the necessary assay sensitivity. Blocking was performed with filtered (syringe filter  $0.2 \mu\text{m}$ , 514-0066, VWR, PA, USA) casein at 1% (w/v) (37582, Thermo Scientific, MA, USA) for 5 min, and each channel was washed for 1 min with 0.01 M PBS. Plasma samples diluted (1:100) in triplicate and IFX concentrations (0, 0.005, 0.01, 0.05, 0.07, 0.1 and  $0.2 \mu\text{g mL}^{-1}$ ) were flowed for 10 min, then the channels were washed with PBS. Anti-human-IgG-HRP (A18823, Invitrogen, MA, USA) at  $50 \mu\text{g mL}^{-1}$  was flowed for 10 min followed by a single wash step.

Luminol solution (SuperSignal™ west femto maximum sensitivity substrate, 34094, Thermo Scientific, MA, USA) was used

as a chemiluminescent substrate for HRP with a flow rate of  $5.5 \mu\text{L min}^{-1}$ , applied with positive pressure using a syringe pump (NE-300, New Era Pump Systems, Inc., NY, USA). The signal acquisition was performed using a Leica DMLM fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DFC300FX digital colour camera. The chemiluminescent images were obtained using Leica Application Suite (version 4.12.0, Leica Microsystems, Wetzlar, Germany), 60 and 90 s after starting to pump the luminol solution, with a digital gain of  $10\times$ , and exposure time of 20 s with  $2 \times 2$  binning. For each microchannel, the chemiluminescence signal was measured using the micrographs taken at 3/4 of the channel length from the inlet using a centred square area with an edge length of 1/3 of the microchannel width, 90 s after start pumping luminol solution. The micrographs of the chemiluminescent signal were quantified using ImageJ software (National Institutes of Health, MD, USA). Data analysis was carried out using Prism 9 (GraphPad Software Inc., CA, USA).

#### Infliximab quantification by indirect well plate ELISA (cELISA)

Corning 96-well half-area clear flat-bottom high bind microplate (CLS3690, Corning Inc. NY, USA) were used to performed IFX quantification. The incubators used were Binder™ KB 720 (Binder, NY, USA) for incubations at  $37^\circ\text{C}$  and INCU-Line 68R (VWR, PA, USA) for incubations at  $24^\circ\text{C}$ . The water used was ultrapure water (Direct-Pure UP Water System, Interlab,



Johnsonville, New Zealand). PBS-T at 0.5% (v/v) was used to remove non-specific binding. All wash steps were performed with the addition of 150  $\mu\text{L}$  of PBS-T to each well. After each last wash, the microplate was dried by tapping upside down on an absorbant paper to remove liquid excess to prevent reagent dilution.<sup>37</sup> Fifty microliters of each reagent were added to perform the cELISA.

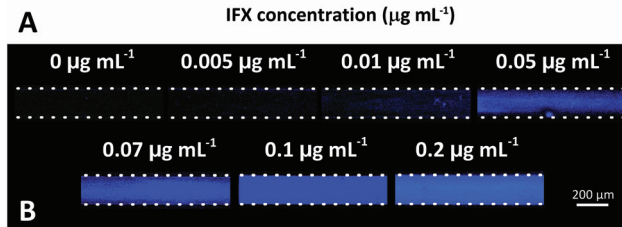
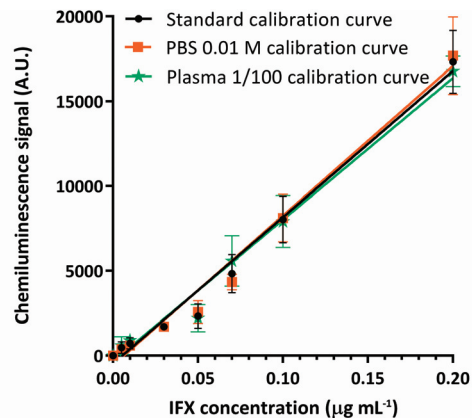
The microplate was coated with 200 ng of TNF- $\alpha$  (300-01A, Peprotech, NJ; USA) and incubated overnight at 4 °C. The following day, the microplate was washed with PBS-T. A solution of 3% (w/v) BSA in PBS-T was used as a blocking agent and incubated for 60 min, at 37 °C, followed by a wash step. Diluted plasma samples (1 : 100) were tested in triplicates and IFX controls (Remicade, Janssen Biotech, Inc., PA, USA) with increasing concentrations (0.001, 0.005, 0.01, 0.03, 0.05, 0.1 and 0.2  $\mu\text{g mL}^{-1}$ ) were added to the microplate to determine the calibration curve of the assay. These were left to incubate for 60 min at 24 °C. Then the wells were washed three times in 150  $\mu\text{L}$  of PBS-T. Each well was then incubated with 1 ng of anti-human-IgG-HRP for 30 min, at 24 °C followed by three washes with PBS-T. To quantify the results, the samples were incubated for 30 min at 24 °C with TMB (613544, Sigma-Aldrich, MO, USA), where the reaction was stopped with 0.5 M  $\text{H}_2\text{SO}_4$ . Finally, the samples were read, by measuring the absorbance at  $\lambda = 450 \text{ nm}$  (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, MA, EUA) and the concentration of IFX was obtained by interpolating the absorbances of the calibration curve. Absorbances values were normalized using negative controls obtained from healthy donors. Data obtained was processed using Microsoft Excel (Microsoft Corporation, NM, USA) and Prism 9.

## Results and discussion

To demonstrate the use of the mELISA as an alternative for POC diagnostics, the IFX concentration in patient samples was determined by both the standard cELISA as well as with the microfluidic immunoassay approach, as it is illustrated in Fig. 1. The in-house cELISA was performed in accordance with the protocol used by the analysis services offered by our laboratory – Molecular Microbiology and Biotechnology (iMed, Lisbon, Portugal).

### Establishing a calibration curve

A standard calibration curve is essential to perform a IFX quantification with lower costs, since it reduces the number of assays to two, sample and blank. To obtain the IFX standard calibration curve, 6 calibration curves of IFX diluted in PBS and 4 curves of IFX diluted in 1/100 plasma were performed, as shown in Fig. 3A. Similar results were obtained using PBS or 1/100 plasma. In all calibration curves, the coefficient of determination varied between 0.967 and 0.993, being accepted in our cELISA values higher than 0.960. The main difference between the use of PBS or plasma is the increase of the background signal in the plasma calibration curve. However, this

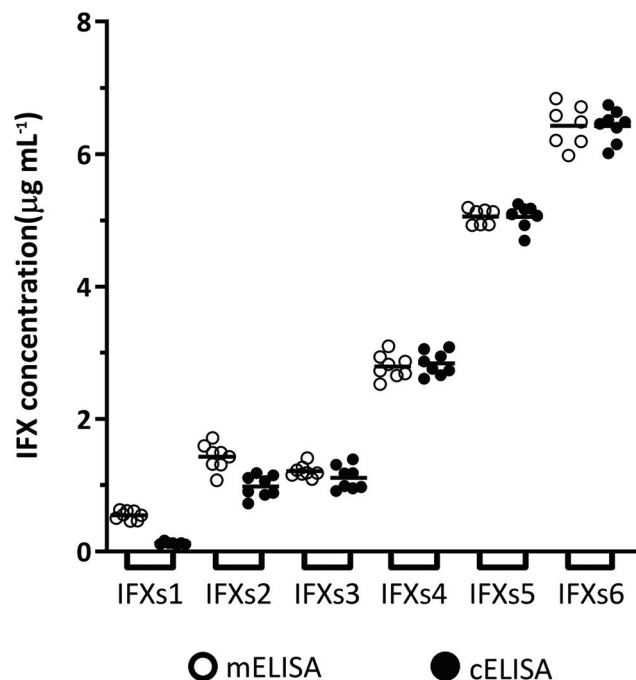


**Fig. 3** Calibration curves of IFX in mELISA. (A) Standard calibration curve obtained using calibration curves of IFX in PBS 0.01 M and in negative plasma diluted 100 times, and (B) representative chemiluminescent signal of the IFX calibration curve in PBS 0.01 M in mELISA. Images were contrast enhanced for visualisation purposes by setting a maximum intensity threshold of 9000 RFU. IFX – infliximab, PBS – phosphate-buffered saline.

difference is negligible when the proper blank is used. Fig. 3B shows micrographs of the chemiluminescent signal obtained for the IFX calibration curve in PBS in mELISA. The limit of blank (LoB;  $\text{mean}_{\text{blank}} + 1.645 \text{ SD}_{\text{blank}}$ )<sup>34</sup> of this immunoassay is shown to be 0.016  $\mu\text{g mL}^{-1}$  ( $n = 22$ ) and the limit of detection (LoD;  $\text{LoB} + 1.645 \text{ SD}_{[\text{lower sample}]}$ )<sup>34</sup> is 0.026  $\mu\text{g mL}^{-1}$ . Taking into account the dilution applied to the samples (1 : 100), the dynamic range of the assay in terms of original sample concentrations is between 2.6 and 20  $\mu\text{g mL}^{-1}$ . These numbers span the complete range of the clinically relevant concentrations (3 to 7  $\mu\text{g mL}^{-1}$ ) which indicates the potential this immunoassay to be used in a clinical setting.<sup>7,16–18</sup> Whereas the commercial POC device has a detection range between 0.4 to 20  $\mu\text{g mL}^{-1}$ , however this total range results from different samples dilution (between 1/20 to 1/200).<sup>28</sup>

### Correlation between different ELISA approaches

In Fig. 4, 6 plasma samples obtained from different patients were tested using both the mELISA and the cELISA approaches. For the mELISA approach, the standard calibration curve was used (Fig. 3), while the cELISA used its own specific calibration curve. The results obtained for both the mELISA and the cELISA are summarized in Table 1, with the values of mean, standard deviations (SD), and coefficients of variation (CV) of the analysed samples. These results indicate that mELISA has a similar precision as the conventional method. In both cases, the quantification of IFX in samples



**Fig. 4** Comparison of IFX quantification in plasma patients' samples by mELISA and cELISA. Plasma patients' samples quantified by cELISA were determined using the calibration curve of the single assay. Samples quantified by mELISA were determined in different days, using the standard curve for calibration. cELISA – conventional ELISA, ELISA – enzyme-linked immunosorbent assay, mELISA – microfluidic ELISA.

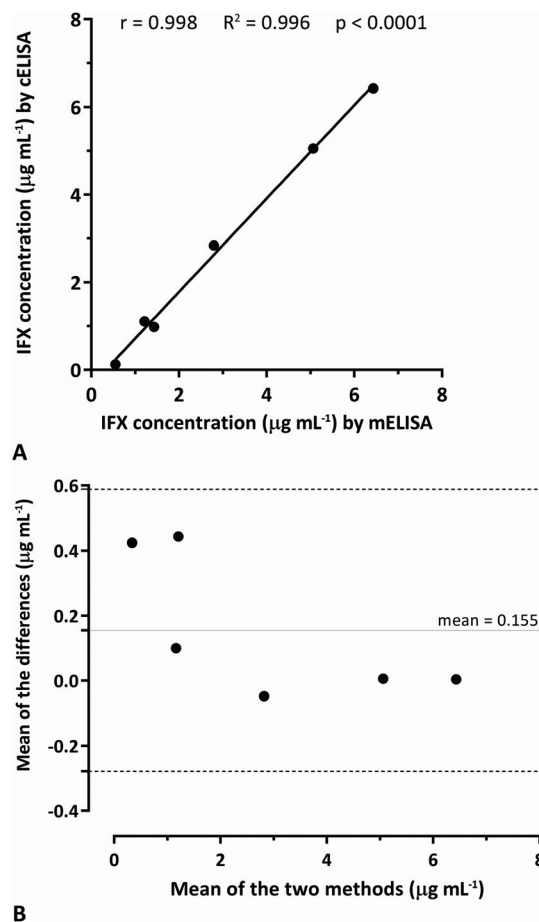
**Table 1** Precision of the conventional and microfluidic ELISA for Infliximab quantification

	cELISA			mELISA		
	Mean (µg mL <sup>-1</sup> )	SD (µg mL <sup>-1</sup> )	CV (%)	Mean (µg mL <sup>-1</sup> )	SD (µg mL <sup>-1</sup> )	CV (%)
IFX s1	0.126	0.020	15.884	0.550	0.067	12.127
IFX s2	0.986	0.163	16.485	1.429	0.196	13.745
IFX s3	1.113	0.178	15.972	1.213	0.094	7.766
IFX s4	2.841	0.178	6.248	2.794	0.179	6.411
IFX s5	5.053	0.186	3.682	5.059	0.116	2.290
IFX s6	6.426	0.241	3.755	6.430	0.313	4.861

cELISA – conventional ELISA, CV – coefficients of variation, ELISA – enzyme-linked immunosorbent assay, mELISA – microfluidic ELISA, SD – standard deviations.

with lower concentration is less precise, as expected, however this issue may be addressed by repeating the analyses with a small dilution, such as performing a dilution of 1/50.

The comparison between the mELISA and cELISA methods to determine the IFX in patient samples was studied using the correlation coefficient and the Bland–Altman plots (Fig. 5). The correlation plot (Fig. 5A) demonstrates that the measurements performed by the cELISA and mELISA are highly correlated, showing a positive correlation ( $R^2 = 0.996$ ;  $p < 0.0001$ ). In the Bland–Altman plot (Fig. 5B) the mean difference between methods is  $0.155 \mu\text{g mL}^{-1}$  with a SD of  $0.221 \mu\text{g mL}^{-1}$ , and



**Fig. 5** Statistical analyses comparing mELISA and cELISA. (A) Correlation plot, and (B) Bland–Altman plot with the representation of the limits of agreement (dotted line) of  $-0.278$  and  $0.588 \mu\text{g mL}^{-1}$ . cELISA – conventional ELISA, ELISA – enzyme-linked immunosorbent assay, mELISA – microfluidic ELISA.

limits of agreement (mean  $\pm 19.6$  SD) of  $-0.278$  and  $0.588 \mu\text{g mL}^{-1}$ . All the mean differences between the two methods are inside these limits, indicating that the mELISA approach can be used to quantify IFX patient samples with the same degree of certainty of the conventional approach.

## Conclusions

In this work, a mELISA with a simple microfluidic design to monitor the IFX therapy in patients, with potential POC applicability is demonstrated. We highlight this method as a simple quantitative approach, with potential to effectively measure IFX concentration directly from patient samples, where the only sample preparation required is a  $100\times$  dilution in 1% (w/v) BSA in PBS, making the mELISA an interesting approach for POC applications. The concentration range of the mELISA is larger than the recommended therapeutic window for IFX therapy. Additionally, the analysis time is 4.5 times shorter than the standard conventional method (*i.e.* cELISA), using the above-described protocols.

As a benchmark for the mELISA, patient samples were tested in both the microfluidic approach and the cELISA, which resulted in very similar results, suggesting a high degree of correlation between the methods ( $r = 0.998$ ;  $R^2 = 0.996$ ;  $p < 0.0001$ ).

The current duration of mELISA is 24 min, requiring an additional 8 min when compared to the lateral flow biosensor approved in Canada (Quantum Blue Infliximab) to quantify IFX. The duration of the mELISA can potentially be further decreased. At this moment our mELISA has a LoD of  $0.026 \mu\text{g mL}^{-1}$ , while the commercial solution provides a significantly higher LoD of  $0.15 \mu\text{g mL}^{-1}$ , indicating that the developed mELISA can detect a IFX concentration 5.8 times lower than the commercial POC product.<sup>28</sup> Towards POC applications, we have also previously demonstrated that this setup is easily amenable to integration with miniaturized photosensors to transduce the chemiluminescence signal.<sup>38</sup> For POC applications, this chip would have to be integrated in a portable system that would provide the external pumping and the optical signal transduction.<sup>39</sup> To avoid the need of an active pumping system, at this moment our group is also designing a capillary microfluidic structure to quantify the IFX, based in our previous work.<sup>40</sup>

The approach developed in this work can potentially be extended to other antibody-based therapeutic monitoring such as ADL or other antibody therapies. The same is also true for the ADAs measurement, which are also essential to adapt the antibody therapy, reducing the symptoms exacerbation on patients and the costs for the healthcare system. However, preliminary studies concerning the ADAs measurement in patient samples showed that the assay lacks the necessary sensitivity. This lack of sensitivity might be resolved by an increase of the adsorption area in the microfluidic device, such as, the incorporation of microbeads to be used as solid supports, improving the surface to volume ratio.

## Author contributions

Inês Iria: validation, formal analysis, investigation, methodology, data curation, writing – original draft, visualization. Ruben R. G. Soares: methodology, writing – review & editing, visualization. Eduardo J. S. Brás: methodology, writing – review & editing, visualization. Virginia Chu: conceptualization, resources, writing – review & editing, supervision, project administration, funding acquisition. João Gonçalves: conceptualization, resources, writing – review & editing, supervision, project administration, funding acquisition. João P. Conde: conceptualization, methodology, resources, writing – review & editing, supervision, project administration, funding acquisition.

## Conflicts of interest

There are no conflicts to declare.

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