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Antigenic response to CT-P13 and infliximab originator in inflammatory bowel disease patients shows similar epitope recognition

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Summary

Aim: To test the cross-immunogenicity of anti-CT-P13 IBD patients' sera to CT-P13/infliximab originator and the comparative antigenicity evoked by CT-P13/infliximab originator sera.

Methods: Sera of patients with IBD with measurable anti-CT-P13 antibodies were tested for their cross-reactivity to 5 batches of infliximab originator and CT-P13. Anti-drug antibody positive sera from treated patients were used to compare antigenic epitopes.

Results: All 42 anti-CT-P13 and 37 anti-infliximab originator IBD sera were cross-reactive with infliximab originator and CT-P13 respectively. Concentration of anti-drug antibodies against infliximab originator or CT-P13 were strongly correlated both for IgG1 and IgG4 ($P < 0.001$). Anti-CT-P13 sera of patients with IBD (n = 32) exerted similar functional inhibition on CT-P13 or infliximab originator TNF binding capacity and showed reduced binding to CT-P13 in the presence of five different batches of CT-P13 and infliximab originator. Anti-CT-P13 and anti-infliximab originator IBD sera selectively enriched phage-peptides from the VH (CDR1 and CDR3) and VL domains (CDR2 and CDR3) of infliximab. Sera reactivity detected major infliximab epitopes in these regions of infliximab in 60%-79% of patients, and no significant differences were identified between CT-P13 and infliximab originator immunogenic sera. Minor epitopes were localised in framework regions of infliximab with reduced antibody reactivity shown, in 30%-50% of patients. Monoclonal antibodies derived from naïve individuals and ADA-positive IBD patients treated with CT-P13 provided comparable epitope specificity to five different batches of CT-P13 and infliximab originator.

Conclusions: These results strongly support a similar antigenic profile for infliximab originator and CT-P13, and point toward a safe switching between the two drugs in anti-drug antibody negative patients.

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1 | INTRODUCTION

An infliximab biosimilar was introduced worldwide and more recently in the USA under the trade name Inflectra or Remsima (developmental name CT-P13, produced by Celltrion, South Korea).[1-3] Infliximab is an anti-tumour necrosis factor (anti-TNF) chimerical monoclonal IgG1 antibody with proven efficacy in IBD, as well as in other immune-mediated chronic inflammatory disorders such as rheumatoid arthritis (RA), psoriasis, psoriatic arthritis and ankylosing spondylitis (AS).[4,5]

Besides high similarity between the reference product (RP) and the biosimilar antibody in physicochemical and biological terms, clinical studies were needed to establish statistical similarity in pharmacokinetics (PK) and efficacy, and to characterise biosimilar immunogenicity and safety. Results of these studies showed that all major physicochemical characteristics and in vitro biologic activity of CT-P13 and the RP were highly comparable.[1,6] However, long-term treatment with therapeutic monoclonal antibodies (mAbs) may induce anti-drug antibody (ADA) formation that is associated with lower drug levels and clinical nonresponse.

Biological therapeutics, particularly complex products such as mAbs, can have numerous quality attributes that can potentially impact immunogenicity, and indirectly, the efficacy of the product. Features important to these functions are known as "critical quality attributes."[7] This knowledge enables scientists to develop high-quality biosimilar products. Critical quality attributes include glyco-heterogeneity and protein aggregation that is determined both by the cell line and manufacturing process, but also by the production process.[8]

All mAbs are immunogenic, that is, can evoke anti-drug antibodies in some patients, likely dependent on the genetic HLA background.[9] The presence of antibodies to infliximab has been associated with higher infliximab clearance, decreased drug serum concentrations, diminished drug efficacy and a higher incidence of adverse events during treatment. As such, guaranteeing immunogenicity similarity between an originator biologic and its biosimilar is a noteworthy reason for concern. Although the rates of anti-drug antibodies to CT-P13 and infliximab originator were comparable in two pivotal trials (where only RA and AS patients were included), there is still limited information relating to cross-immunogenicity of infliximab and CT-P13, especially in patients with IBD.[10-12] As reported by Ben-Horin et al, antibodies against infliximab in patients with IBD bind and functionally inhibit CT-P13 to a comparable degree, anticipating equivalent immunogenicity and shared immunodominant epitopes on these two infliximab versions.[13] The immunogenic response against the biosimilar and the original drug is the most sensitive evaluation of biosimilarity in vivo. However, to complete the immunogenicity equivalence, it is also important to evaluate the opposite, ie, to determine if patient-derived anti-drug antibodies against CT-P13 cross-react with RP.[14,15] In this exercise, it is important to compare not only the quantity but also the quality of anti-infliximab antibodies. This result will help to confirm whether new epitopes are created in biosimilar infliximab compared to the originator and the impact of these antibodies on the activity of infliximab.

This study aimed to address the feasibility of therapeutic switch between infliximab and CT-P13. The antigenic comparability between infliximab and CT-P13 was tested by examining if antibodies elicited by CT-P13 treatment in patients with IBD also cross-reacted and recognised infliximab to a similar degree. To further consolidate the equivalent immunogenicity between the two versions of infliximab we have characterised the epitopes recognised by anti-infliximab antibodies and compared them between CT-P13 and infliximab.

2 | METHODS

2.1 | Study population

We included in this study, sera from 99 patients with IBD from Portugal, the Netherlands, Israel, Italy, and Hungary, treated with CT-P13 or infliximab (Table 1). IBD was diagnosed by the combination of established clinical, radiological, and endoscopic criteria. The sera of 20 infliximab-treated RA patients without detectable antibodies against CT-P13 and infliximab and sera of healthy individuals were used in this study as control sera. For infliximab-treated patients, sera were obtained before the next infusion, and trough level of the drug and anti-drug antibodies were determined by indirect and bridging ELISA respectively. Patients treated with CT-P13 were both experienced and naïve for infliximab, but all were negative for infliximab ADAs at the beginning of CT-P13 treatment.

2.2 | Measurement of ADA cross-reactivity with infliximab and CT-P13

To assess whether anti-CT-P13 antibodies from patients with IBD cross-reacted with infliximab, we measured the concentration of anti-drug antibodies in sera. Negative controls (ADA-negative) consisted of patients with IBD unexposed to infliximab, RA patients without detectable anti-infliximab antibodies and healthy individuals. These samples are part of a biobank control sera from our laboratory and were validated with different immunogenicity tests. Anti-drug antibodies were determined by bridging ELISA with acidic treatment, which is also capable of detecting ADAs in the presence of the drug. Briefly, infliximab (0.5 mg/mL) was added to ELISA plates (Nunc, Denmark). We used five different batches of infliximab (2RMKA81202, 2RMKA82501, 4RMA67004, 4RMA72302, 4RMA74203) and CT-P13 (12B1M001FB1, 2857113, 12B2M001FB1, 14B1M014FB1,2059124) that were circulating in Portugal to compare the cross-reactivity between all batches. In all experiments, infliximab (Janssen, USA) and CT-P13 (Celltrion, South Korea) were used in the ELISA plate wells side-by-side for comparison. Serum was added and incubated for 60 min at room temperature. After washing, plates were incubated with biotin-labelled infliximab for 1 hour, at room temperature followed by addition of streptavidin-HRP (Thermo Scientific, USA). The reaction was developed with TMB (Thermo Scientific, USA) substrate and stopped with 2M H_2SO_4. Absorbances were read at 450/540 nm, and the results were

TABLE 1 Clinical characteristics and ADA levels in sera from patients treated with CT-P13 and infliximab

CT-P13-treated patients with IBD and positive ADA (Total number n = 42)	
Anti-TNF Naïve IBD patients (n = 26)[a]	
Origin: Portugal (n = 6); Hungary (n = 9); Italy (n = 10); Netherlands (n = 1)	
Concomitant immunomodulators	Yes
Dose of IFX	5 mg/Kg/8 wk
IFX trough level bellow 1 μg/mL	75%
Anti-TNF experienced IBD patients (n = 16)[b]	
Hungary (n = 5); Italy (n = 11)	
Concomitant immunomodulators	Yes
Dose of IFX	5 mg/Kg/8 wk
IFX trough level bellow 1 μg/mL	79%
Infliximab-treated patients with IBD and ADA-positive (n = 37)	
Origin: Portugal (n = 10); Italy (n = 15); Israel (n = 28)	
Concomitant immunomodulators	Yes
Dose of IFX	5 mg/Kg/8 wk
IFX trough level bellow 1 μg/mL	73%

ADA, anti-drug antibodies; IBD, inflammatory bowel disease; IFX, infliximab; TNF, tumour necrosis factor.
[a]Anti-TNF naïve IBD patients refer to patients treated for the first time with infliximab.
[b]Anti-TNF experienced IBD patients refer to patients previously treated with infliximab.

expressed as μg/mL after normalisation using a standard curve of mouse anti-human antibody (Abcam, UK). The assay's cut-off level of detection for anti-infliximab antibodies was 1 μg/mL.

2.3 | Measurement of IgG4 anti-drug antibodies in the sera

Sera samples containing ADAs for CT-P13 and infliximab were compared for the presence of IgG4. The levels of these antibodies were assessed using an ELISA adapted for IgG4 detection.[16] In this assay, pre-coated plates with the anti-IgG4 antibody (Thermo Scientific, USA) were incubated with 1/10 diluted ADA. After washing, plates were incubated with biotin-labelled infliximab for 1 hour, at room temperature followed by addition of streptavidin-HRP. The reaction was developed with TMB substrate and stopped with 2M H_2SO_4. Absorbances were registered at 450/540 nm, and the results were expressed as μg/mL after normalisation using a standard curve of mouse anti-human antibody.

2.4 | Inhibition of infliximab and CT-P13 binding capacity to TNF

An inhibition assay was performed to assess whether anti-CT-P13 antibodies elicited in IBD patients inhibited to similar extent infliximab and CT-P13 TNF binding capacity.[12] Sera were diluted 1:20 in bovine serum albumin (BSA), preincubated with high concentrations of exogenous infliximab or CT-P13 (10 μg) for 30 min at room temperature, and then added to pre-plated TNF ELISA plates. Horseradish peroxidase activity measured the concentration of the bound infliximab (infliximab originator or CT-P13) labelled anti-human goat F(ab')2 fragment antibody (MP Biomedicals, USA). The reaction was developed with TMB substrate and stopped with 2M H_2SO_4. Absorbances were registered at 450/540 nm, and the results were expressed as μg/mL after normalisation using a standard curve of mouse anti-human antibody.

2.5 | Naïve Antibody Library construction

The Fab lambda (λ) naïve library was constructed as described with slight modifications.[17] RNA was isolated from Peripheral Blood Mononuclear Cells (PBMCs) from fresh blood of 18 healthy volunteers, and the primers used for PCR amplification of human heavy (HC) and light-chain (LC) V-regions, were constructed according to the literature.[17] All primary PCRs were carried out with separate reverse primers and combined forward primers, to maintain maximal diversity. DNAs were amplified using 16 different oligonucleotide primers for the coding regions of the LC and 18 different primers for the HC. A two-step cloning strategy was used to construct the library. First, the LC PCR products were pooled and sub-cloned into the phagemid vector pComb3X (The Scripps Research Institute, USA), giving origin to the lambda (λ) sub-library. Next, the HC pooled fragments were inserted in the sub-library vector creating the final Fab and lambda (λ) Fab library. The resultant ligation product was electroporated into freshly prepared electrocompetent *Escherichia coli* TG1 cells. The library was stored at −80°C as glycerol stock, rescued (using M13K07 as helper phage) and used for phage production according to standard protocols. Plaque assay determined the titre of the primary and amplified library. The Fab lambda (λ) naïve library has 1.29×10^{10} individual clones, 86% diversity, and PCR showed that more than 70% of the phages contained the correctly cloned Fab fragments.

2.6 | Infliximab peptide phage library construction

A phage-display library of infliximab peptides was constructed using the vector pComb3X.[18] For phage surface expression, DNA fragments are required to be cloned in frame with the pelB leader peptide and the gene III phage coat protein present in pComb3 at the N- and C-terminal respectively. Full-length infliximab cDNA was prepared by gene synthesis to construct infliximab cDNA fragment library in pComb3X (Geneart, Germany). Agarose gel electrophoresis separated the 1440 and 650 bp infliximab HC and LC gene fragments and these were purified using a Wizard PCR Preps DNA Purification System (Promega, UK). Digestion with DNAse I prepared random 50-200 bp fragments of infliximab cDNA in a reaction containing: 1 mg cDNA, 1 unit DNAse I (Promega, UK), and DNAse I buffer (Promega, UK). The reaction was incubated at room temperature for 15 minutes and was terminated by the addition of 50 mM

EDTA (pH 8.0). The DNA fragments were purified using a Wizard PCR Preps DNA Purification System and treated with T4 DNA polymerase (Promega, UK) according to the manufacturer's protocol to create blunt ends. After further purification, the fragments were ligated into the EcoRV restriction site of pComb3 using standard methods. The infliximab cDNA fragment library was recovered by electroporation of *E. coli* XL1-Blue cells (Stratagene, USA).[19]

2.7 | Panning of Infliximab peptide Phage-Display

For biopanning experiments, 14 samples of anti-CT-P13 and 10 samples of anti-infliximab patient sera were applied to the wells of Nunc polystyrene 96-well microtitre plates (Nunc, Denmark).[20] Plates were incubated at room temperature for 2 hours to allow antibody binding before washing with 0.05% PBS-Tween 20 (v/v). Plates were incubated for 1 hour to allow the interaction of anti-drug antibodies with peptides displayed on the surfaces of the VCMS13 phage particles. The wells were washed extensively with PBS/Tween to remove unbound phage. Bound phages were eluted with trypsin for 30 minutes. Two aliquots of the infected cells were plated onto a selective medium to allow the recovery of individual bacterial clones for analysis. Phagemid DNA (50 ng samples) was subjected to PCR amplification to confirm the presence of a cDNA insert. The PCR products were analysed by agarose gel electrophoresis and purified according to a Wizard PCR Preps DNA Purification System. Insert DNA sequences were compared with the full-length infliximab cDNA sequence using the BLAST network service of the National Center for Biotechnology Information (Bethesda, MD, USA).

2.8 | Phage ELISA

Phages displaying infliximab peptides required for analysis in phage ELISA were prepared from individual bacterial clones as described elsewhere.[20] Wild-type VCMS13 helper phages were included in each assay as a control for background antibody binding. Wells were washed with 0.1% PBS-Tween 20 (v/v), blocked with 3% (w/v) BSA in PBS at room temperature for 1 hour, and then washed with 0.1% PBS-Tween 20 (v/v) Tween 20. Polyclonal anti-infliximab antibodies were analysed at a dilution of 1:1000. The plates were incubated at room temperature for 2 hours and then washed four times with 0.1% PBS-Tween 20 (v/v). All sera were tested in triplicate and the average OD_{405} values taken. OD_{405} values were corrected for background reactivity to helper phage to give an antibody (Ab) index. The upper limit for negative control was calculated using the mean Ab index of three standard deviations of the population of 20 healthy individuals. Any sample with an Ab index above the upper limit of normal was designated as positive for antibody reactivity to the phage-displayed infliximab peptide.

To prepare Fab and Fc, we used immobilised papain protease to digest infliximab and CT-P13 and subsequently purified the Fab and Fc domains using Protein A agarose (GE, Sweden). Protein concentration was determined with a BCA assay (ThermoFischer, Germany), and the monomer fraction was used for ELISA as described above.

2.9 | Synthetic peptide ELISA

Synthetic peptides were synthesised (GeneCust, Belgium). We coated high-affinity polystyrene 96-well microtitre plates (Nunc Immobilizer Amino F96, Denmark) with 10 μg of the peptide in 3.5 mM $NaHCO_3$ (pH 9.2) and incubated overnight at 4°C. We performed the peptide ELISA with anti-CT-P13, anti-infliximab and control sera at dilutions of 1:50. The plates were incubated at room temperature for 2 hours and then washed four times with 0.1% PBS-Tween 20 (v/v). Antibody binding was detected with alkaline phosphatase-conjugated anti-human Fc mAb. All sera were tested in triplicate, and the average OD_{405} value was taken to give an Ab index. The upper limit of normal for each assay was calculated using the mean Ab index with three standard deviations (SD) of the population of 10 healthy individuals. Any sample with an Ab index above the upper limit of normal was designated as positive for antibody reactivity to the infliximab peptide.[17,18]

2.10 | Construction of antibody libraries against CT-P13

We obtained three blood samples from IBD patients with positive ADA for CT-P13, and the sera were titrated by bridging ELISA. The concentration of anti-CT-P13 antibodies was expressed in ng/mL. We used phage-display DNA vector pComb3X for human Fab library construction and propagation in *E. coli* strain XL1-Blue (Agilent, USA).[21] For lymphocyte RNA preparation and human Fab antibody library construction, Ficoll-Hypaque gradient (GE, Sweden) separated B lymphocytes from donors, and total RNAs from each sample was extracted using the QIAamp Blood Mini Kit (Qiagen, Germany). cDNA was synthesised using the SuperScript Pre-amplification System (Thermo Scientific, USA) and used as a template for PCR amplification of the human light and heavy gene fragments. A phage library displaying human Fab antibodies was constructed in pComb3X according to published protocols with minor modifications.[20] Biopanning of the Fab library was carried out on purified CT-P13 (Celltrion, South Korea) using standard protocols.[20] For phage ELISA, single colonies of anti-CTP13 Fab clones were used to grow phage-Fabs in three 96-well culture plates followed by superinfection with diluted VCSM13 helper phage. The bound phages-Fabs were detected with an HRP-conjugated anti-M13 antibody (diluted 1:5000) (GE, Sweden). We repeated the ELISA at least twice. Human Fabs which bound specifically to CT-P13 were defined by having at least a threefold signal over background and in the large majority of cases >5-fold higher than that seen on irrelevant control antigen.[20]

2.11 | Statistical analysis

Data analyses, including the statistical assessment of similarity, were performed taking into consideration the principles/issues discussed in applicable guidelines.[19] As required by the study goals and also addressed in the applicable guidelines, the recommended type of comparison performed is related to the

similarity/equivalence claim. Therefore, we used two-sided one-sided tests (TOST) for equivalence assessment. The adopted acceptance criteria for declaring equivalence are those included in the EMA "Investigation of bioequivalence" guideline. In this guideline, a 90% CI (corresponding to one-sided test using an $\alpha = .05$) for the ratio of the test and reference products of the parameters/endpoints under study should be contained within the acceptance interval of 80%-125%. The number of observations in all comparisons made was larger than that required to ensure a statistical power of 80%. A significant statistical equivalence was accepted whenever the estimated *P*-value was smaller than the pre-specified alfa error. Due to the underlying theoretical assumptions, the parametric tests require that some conditions are fulfilled. For instance, the normality distribution of the data for the continuous variables was tested using the Shapiro-Wilk test and the observation of Q-Q plots. The coefficients of the correlation of the numerical attributes measured for the RP and the biosimilar were determined using the nonparametric Spearman's correlation test. The prevalence of antibody reactivity to phage-displayed and synthetic infliximab peptides was compared between patient groups and controls using Fisher's exact test. Intra- and inter-assay variations were calculated as percentage coefficients of variation. Differences in antibody titres were analysed by the Wilcoxon matched-pairs test. The GraphPad Prism (v.7.0) and the TOSTR package software were used to perform the appropriate statistical tests.

3 | RESULTS

3.1 | Cross-reactivity of anti-CT-P13 antibodies with infliximab

In the first experiment, we tested 42 samples with anti-drug antibodies from IBD patients treated with CT-P13 for more than 9 months (Table 1). These patients were treated in Portugal, the Netherlands, Italy, and Hungary with CT-P13. From the individual 42 ADA-positive sera used in this study, 16 samples were obtained from patients previously exposed to infliximab. Whenever feasible, side-by-side paired experiments were performed to decrease the effect of the uncontrolled experimental variability and therefore increase the statistical power. As such, after defining the study goals, a major effort was made to define the most appropriate, yet feasible, set of experiments that would generate the data considered critical to conclude (or not) on the antigenic similarity. A robust experimental design required that any difference between products was not confounded with the inherent and unavoidable inter-batch differences and that the inter-product similarity was comparable to the inter-batch variability. This assessment required that several different batches of infliximab and CT-P13 be used. In this particular work, the attributes considered to be critical to demonstrate the antigenic similarity (if true) were both quantitative and qualitative.[22]

Initially, we tested whether anti-drug antibodies elicited to CT-P13 would cross-react with five different batches of infliximab and CT-P13. One time point ADA-positive sample per patient was used for cross-reactivity between CT-P13 and infliximab. In this experiment, we analysed sera from ADA-positive IBD patients as well as 20 negative control sera from ADA-negative RA patients and healthy individuals to eliminate background signal in the ELISA assay. As shown in Figure 1A,B, all 42 ADA-positive sera of CT-P13 sensitised patients were cross-reactive with all batches of infliximab and CT-P13. These batches are representative of CT-P13 used in Portugal during 2015 and 2016. In addition, we observed a similar antibody cross-reactivity between sera from patients pre-exposed to infliximab and anti-TNF naïve (data not shown). The cross-reactivity observed between five different batches of CT-P13 and infliximab exhibited a good correlation between anti-CT-P13 and anti-infliximab ADA titres (with $r \geq .98$, 95% CI 0.97-0.99, $P < 0.001$ for all comparisons, Spearman's correlation test, Figures 1A,B, and Data S1). In addition, the correlation between anti-CT-P13 and anti-infliximab antibodies was not different from the correlation between different batches of CT-P13 (Figures S1-S3).

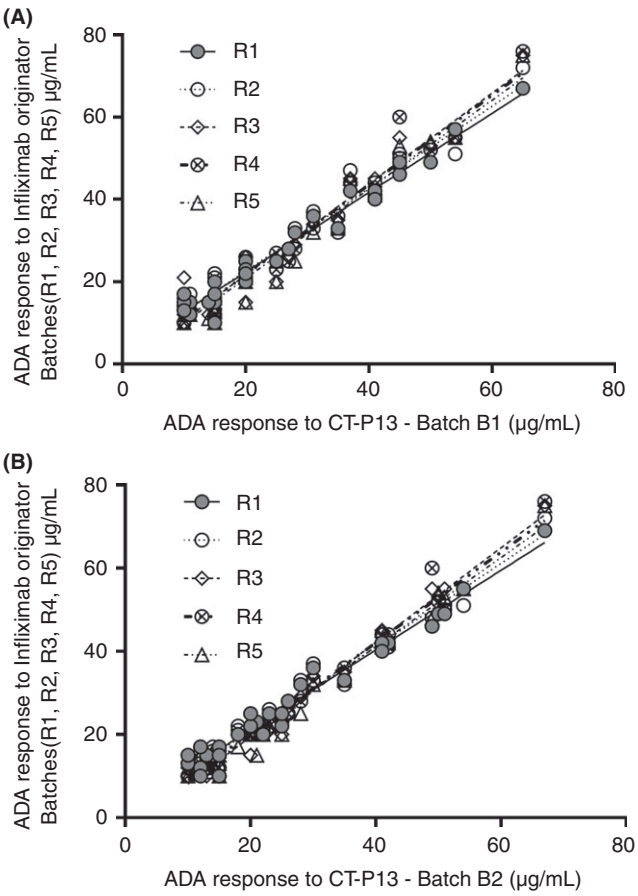

FIGURE 1 A, B, Dot plots of correlations between levels of anti-CT-P13 antibodies and anti-infliximab antibodies in ADA-positive sera (n = 42). Cross-reactivity is indicated with two batches of CT-P13, and five batches of infliximab and each marker in the graph indicates the value obtained for a single patient serum. The remaining dot-plot correlations for other three CT-P13 batches are shown in supplementary figures. Pearson correlation was calculated with estimated confidence intervals and *P*-values

To further investigate whether the ADA response against CT-P13 was similar to infliximab, we tested ADA-positive sera samples for quantification of IgG4 against both versions of infliximab (Figure 2). This corroboration used 42 samples from patients with IBD treated with CT-P13 and 20 control samples from ADA-negative sera. We tested these samples against five different batches of CT-P13, along with five different batches of infliximab. The results showed a similar response of IgG4 against different batches of infliximab (9.10 ± 0.18 μg/mL, n = 185) and CT-P13 (9.40 ± 0.20 μg/mL, n = 210) products (d = 0.30, Cohen's d = 0.183, df = 393, $P < 0.001$). Despite the small difference (0.30 μg/mL, equivalent to 3.3%) and with no clinical relevance, a low P-value (P = 0.069) results from the statistical comparison between the two anti-CT-P13 IgG4 averages (two-tailed t test; ANOVA two way with replication, df = 393) (Figure 2). This variation was detected for both versions of infliximab and might originate from the IgG4 detection methodology. In ADA-positive sera from patients that switched from infliximab to CT-P13, the mean titre of anti-drug IgG4 between the infliximab batches (9.05 ± 0.40 μg/mL, df = 80) and CT-P13 batches (9.28 ± 0.49 μg/mL, n = 80) was found to be similar (d = 0.22, Cohen's d = 0.109, df = 80, $P < 0.001$) suggesting that comparable epitopes for both infliximab drugs are recognised by the two classes of IgG. These results seem to indicate that the concentrations of IgG4 in sera elicited against CT-P13 recognise similarly CT-P13 or infliximab versions.

3.2 | Functional inhibition of infliximab and CT-P13 binding to TNF by anti-CT-P13 patient sera

We tested 32 sera samples with anti-CT-P13 activity in a binding-inhibition assay to investigate if polyclonal anti-drug antibodies formed in CT-P13-treated IBD patients could functionally inhibit the binding of different batches of CT-P13 or infliximab to TNF. These IBD patient sera were chosen based on their low infliximab trough levels. Due to the reduction in infliximab pharmacokinetics these samples might be considered as containing anti-CT-P13 neutralising antibodies. As shown in Figure 3, anti-CT-P13 positive sera inhibited with similar extent the binding to TNF-α of different infliximab batches (24.7 ± 0.8%, n = 80) and CT-P13 batches (25.6 ± 0.8%, n = 80). These results were statistically equivalent (d = 0.85, Cohen's d = 0.201, df = 158, $P < 0.001$). Conversely, ADA-negative sera showed no inhibitory effect on the binding capacity of infliximab or CT-P13 to TNF, which was similar to the independent binding of both drugs to the target (Figure 3). Subgroup analysis of IBD patients previously exposed to infliximab showed a similar neutralisation activity of CT-P13 and infliximab binding to TNF compared to infliximab naïve sera (data not shown). The sera elicited to CT-P13 also showed no inhibition toward the binding of adalimumab and etanercept to TNF. The binding of adalimumab and etanercept to TNF was similar in the presence and absence of anti-CT-P13 sera. These results seem to indicate that anti-drug antibodies developed against CT-P13 recognised specifically infliximab-derived epitopes and not other anti-TNF antibodies, suggesting that CT-P13 did not elicit antigenic responses different to infliximab originator.

3.3 | Comparative mapping of infliximab antigenicity by anti-infliximab originator and anti-CT-P13 IBD patient sera

To define the binding sites of anti-CT-P13 polyclonal antibodies from IBD patients, we employed the phage-display technology. Having set this objective, we constructed a library of randomly generated infliximab DNA fragments in the phage-display vector pComb3X. The size

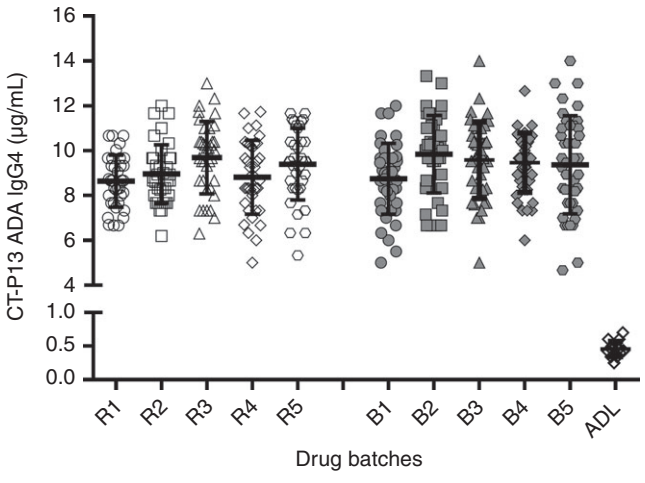

FIGURE 2 Measurement of IgG4 in anti-CT-P13 and anti-infliximab sera (n = 42) toward five CT-P13 and infliximab batches. Reactivity against adalimumab was also examined for each serum. Each marker denotes the result obtained for a single serum sample. The P-value indicates differences between infliximab and CT-P13 batches in total

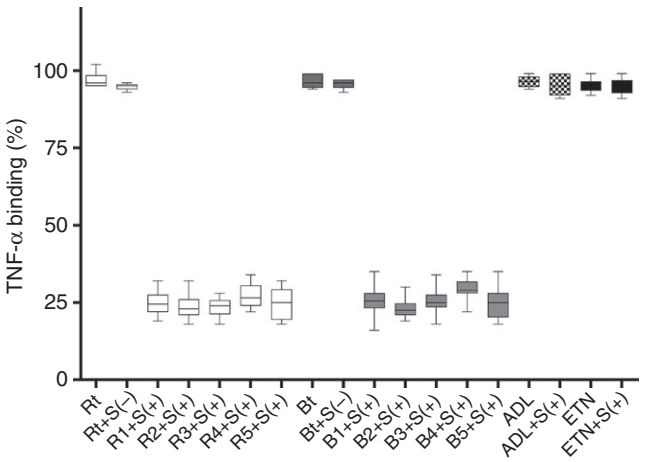

FIGURE 3 Five batches of CT-P13 (B1 to B5) and infliximab (R1 to R5) TNF binding capacity is inhibited similarly in the presence of anti-CT-P13 neutralising antibodies (n = 32). (Rt) and (Bt) denote a mixture of infliximab and CT-P13 batches respectively. (Se) denotes patient sera samples. Adalimumab and etanercept were used as negative controls for the binding of anti-infliximab sera. TNF binding is represented as a percentage of drug binding without the presence of serum

of the infliximab DNA fragment library was 2×10^5 independent clones. Amplification by PCR of phagemid DNA isolated from individual bacterial clones demonstrated that the infliximab cDNA fragment library was 99% recombinant, with insert size ranging from less than 100 to 200 bp. The sequencing of individual phagemid DNA indicated that the library contained random infliximab cDNA fragments with no bias for any particular infliximab sequences. We immunoscreened the infliximab phage-display library in biopanning experiments with 14 anti-CT-P13 and ten anti-infliximab sera patients, together with three control sera. Our goal was to enrich immunoreactive peptides recognised by anti-CT-P13 and anti-infliximab antibodies. Four rounds of biopanning were carried out, and phagemid DNA from 20 to 40 bacterial clones were analysed by DNA sequencing to identify infliximab peptides that were selected during the enrichment process. Comparison of the consensus peptides enriched by anti-CT-P13 and anti-infliximab sera enabled identification of consensus sequences enriched in different patients and identified six heavy-chain (HC) fragments, four light-chain (LC) and three constant HC peptides with biopanning against polyclonal anti-CT-P13 (Tables 2 and 3). Likewise, when we panned the infliximab library against anti-infliximab polyclonal sera similar peptide regions were enriched. To confirm immunoreactivity to phage-displayed infliximab peptides identified in biopanning experiments, sera from 42 anti-CT-P13, 37 anti-infliximab patients and nine healthy controls were tested in a phage ELISA format. All sera were analysed against all peptide regions identified in biopanning. We calculated an Ab index for each serum, and the upper limit of normal for each phage ELISA was determined (Figure 4). Any serum sample with an Ab index above the upper limit of normal was designated as positive for antibody reactivity to the phage-displayed infliximab peptide. The antibody indices for anti-CT-P13 and anti-infliximab patient sera, as well as control sera are shown in Figure 4. Intra- and inter-assay variations for each sample were no more than 10% and 12% respectively.

From the 42 anti-CT-P13 IBD patient sera analysed, 38%-83%, 48%-83%, and 38%-48% detected variable heavy-chain (VH), variable light-chain (VL) and constant heavy (CH) regions respectively. Using 37 samples of anti-infliximab sera, these same regions of infliximab were detected by 30%-48%, 48%-81% and 68%-86% respectively (Tables 2 and 3). All control sera were negative for antibody reactivity against all tested phage-displayed infliximab peptides. We were not able to detect any phage-displayed infliximab peptides in four and two patient samples from anti-CT-P13 to anti-infliximab respectively. We cannot exclude that the sera from these patients recognised different epitopes or conformational regions in infliximab which were not enriched in biopanning. Antibodies against infliximab peptides 39-81 in the VH, 1-39 and 50-97 in the VL, were significantly more prevalent in anti-CT-P13 and anti-infliximab patients compared with controls ($P < 0.001$, P¼.0072 and P¼.0216, respectively; see Table 3). Surprisingly, we have observed a predominant binding of anti-CT-P13 and anti-infliximab patient sera to the peptide that encompasses amino acids 177-207 in the CH region. Nevertheless, we do not observe significant differences between anti-CT-P13 and anti-infliximab polyclonal antibody binding to all enriched infliximab regions. When we compared anti-CT-P13 sera from pre-exposed infliximab patients and anti-TNF naïve patients we observed a similar recognition of infliximab regions (data not shown). These results seem to indicate that immunogenic antibodies raised in IBD patients against CT-P13 and infliximab recognise linear and conformational epitopes similarly in the two versions of infliximab.

The apparent multiple sera binding sites in infliximab peptide regions may be conformational or linear in phage ELISA. To confirm antibody reactivity in phage ELISA, and to refine the comparison between anti-CT-P13 and anti-infliximab antigenic response in patients, we used synthetic 15 amino acid peptides (12 peptides for the HC region and nine peptides for the LC region) that are encoded in all infliximab phage-display regions (Table 4 and Figure S5-S6). These synthetic peptides encompassed all antigenic regions enriched

TABLE 2 Infliximab consensus peptide sequences enriched in biopanning experiments

Samples used in biopanning experiments	Infliximab peptides sequences	Amino acid residues of IFX	Number of clones with sequence (%)
A-CTP13/A-Rem	KLEESGGGLVQPGGSMKLS	VH	7/20 (35)
A-CTP13/A-Rem	SGGGLVQPGGSMKLSCVASGFIFSNHWMN	VH	11/20 (55)
A-CTP13/A-Rem	SGFIFSNHWMNWVRQSPEKGLEWVAEIRSRS	VH	24/36 (67)
A-CTP13/A-Rem	QSPEKGLEWVAEIRSKSINSATHYAESVKGRFTISRDDSKSAV	VH	21/30 (70)
A-CTP13/A-Rem	AESVKGRFTISRDDSKSAVYLQMTDLRTEDTGVYYCSRNYY	VH	20/40 (50)
A-CTP13/A-Rem	DLRTEDTGVYYCSRNYYGSTYDYWGQGTTLTVS	VH	12/25 (48)
A-CTP13/A-Rem	DILLTQSPAILSVSPGERVSFSCRASQFVGSSIHWYQQR	VL	16/35 (46)
A-CTP13/A-Rem	ERVSFSCRASQFVGSSIHWYQQRTNGSPRLLIKYASESMSGGIPSRFS	VL	12/25 (48)
A-CTP13/A-Rem	YASESMSGIPSRFSGSGSGTDFTLSINTVESEDIADYYCQQSHSWP	VL	18/30 (60)
A-CTP13/A-Rem	ESEDIADYYCQQSHSWPFTFGSGTNLESW	VL	12/30 (40)
A-CTP13/A-Rem	LSSVVTVPSSSLGTQTYICNVNHKPSNTKVD	CH1	5/30 (17)
A-CTP13/A-Rem	PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED	CH2	6/30 (53)
A-CTP13/A-Rem	NWYVDGVEVHNAKTKPREEQYNSTYRVVSVL	CH2	8/30 (27)

CH, constant heavy region; IFX, infliximab; VH, variable heavy-chain region; VL, variable light-chain region.

TABLE 3 Results of Phage ELISA

Infliximab peptide sequences displayed on phage	Nr	Number of patients infliximab ADA sera positive in phage ELISA	Number of patients CT-P13 ADA sera positive in phage ELISA	Number of control sera positive for antibody reactivity in phage ELISA	*P*-value
VH (aa 3-19)	1	18/37 (48%)	24/42 (57%)	1/20	<0.001
VH (aa 7-35)	2	25/37 (73%)	29/42 (69%)	0/20	<0.001
VH (aa 25-55)	3	26/37 (70%)	29/42 (69%)	0/20	<0.001
VH (aa 39-81)	4	27/37 (73%)	30/42 (71%)	0/20	<0.001
VH (aa 63-97)	5	30/37 (81%)	33/42 (79%)	0/20	<0.001
VH (87-119)	6	29/37 (77%)	34/42 (81%)	0/20	0.0072
CH1 (aa 177-207)	7	18/37 (48%)	19/42 (45%)	0/20	0.003
CH2 (aa 268-300)	8	16/37 (43%)	16/42 (38%)	0/20	<0.01
CH3 (aa 306-336)	9	20/37 (30%)	20/42 (48%)	1/20	<0.001
VL (aa 1-39)	10	27/37 (73%)	31/42 (74%)	0/20	<0.001
VL (aa 17-63)	11	26/37 (86%)	33/42 (79%)	0/20	<0.001
VL (aa 50-97)	12	30/37 (81%)	35/42 (83%)	1/20	<0.01
VL (aa 79-107)	13	29/37 (68%)	31/42 (73%)	1/20	<0.01

CH, constant heavy region; ELISA, enzyme-linked immunosorbent assay; VH, variable heavy-chain region; VL, variable light-chain region.

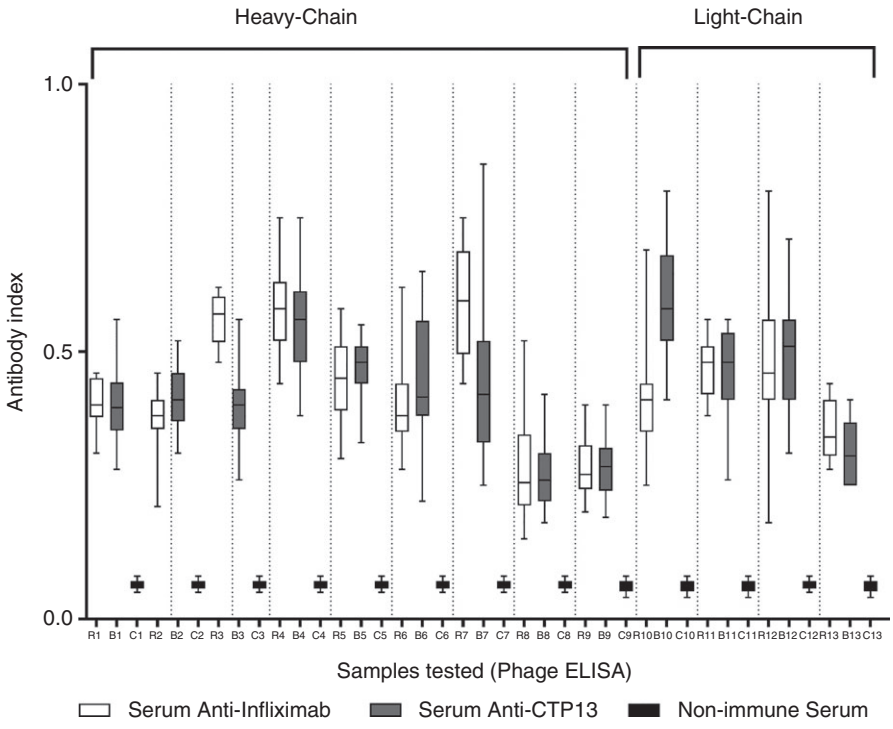

FIGURE 4 Phage ELISAs with patient and control sera. Patient and control sera were analysed for antibody binding in phage ELISA, as detailed in Methods section. The antibody indices are shown for patients treated with CT-P13 (B) (n = 42) and infliximab (R) (n = 37) and for control (C) (n = 20) non-immune serum samples in phage ELISAs against phage-displaying infliximab peptides. Antibody index is calculated by the elimination of background sera binding. The Heavy-chain and Light-chain localisation on infliximab were obtained from the ImMunoGeneTics information system (IMGT) and the Crystal Structure Of TNF-alpha In Complex With infliximab Fab (PDB: 4G3Y_H)

in biopanning and were used to compare the antibody response of anti-CT-P13 and anti-infliximab sera to peptide epitopes. We used an irrelevant control peptide of HIV-1 in all ELISA experiments. Antibody indices and the mean percentage antibody binding were calculated for each sera sample.

As shown in Figure 5A,B, relative immunoreactivity of anti-CT-P13 and anti-infliximab sera against all peptides was similar for all patient samples tested. Control sera were negative for antibody reactivity against all synthetic infliximab peptides. We observed a reduction in the percentage of patients' sera that recognised HC and LC linear peptides compared to phage-encoded infliximab regions (Table 4). As shown in Figure 5A, the peptides predominantly recognised by anti-CT-P13 and anti-infliximab in VH of infliximab are H2 (aa 25-55) and H7, H8, H9 (aa 63-119). The combination of these epitope regions consists of a paratope structure of the HC (CDR1 and CDR3) responsible for the binding to TNF. Nevertheless, these epitope peptides showed a reduced binding reactivity to IBD patient sera and revealed a mixed prevalence of sera recognition. The epitopes H2 were recognised by 38% of CT-P13 and infliximab patient sera, and H7 and H9 showed a higher prevalence (H7, 70% vs 67%

TABLE 4 Epitope specificities of IBD patient anti-CT-P13 and anti-infliximab antibodies

Phage peptide region	Infliximab peptide epitopes	Number of infliximab patients with ADA recognising epitope	Number of CT-P13 patients with ADA recognising epitope	*P*-value
VH (aa 3-19)	H1-GGSMKLSCVASGFIF	14/37 (37%)	16/42 (38%)	<0.01
VH (aa 25-55)	H2-NHWMNWVRQSPEKGL	14/37 (38%)	16/42 (38%)	<0.01
VH (aa 25-55)	H3-WMNWVRQSPEKGLEW	21/37 (57%)	25/42 (60%)	<0.01
VH (aa 39-81)	H4-LEWVAEIRSKSINSA	26/37 (70%)	29/42 (69%)	<0.05
VH (aa 39-81)	H5-ATHYAESVKGRFTIS	25/37 (68%)	30/42 (71%)	<0.05
VH (aa 63-97)	H6-SRDDSKSAVYLQMTD	23/37 (62%)	25/42 (60%)	<0.001
VH (aa 63-97)	H7-LQMTDLRTEDTGVYY	26/37 (70%)	28/42 (67%)	<0.01
VH (87-119)	H8-EDTGVYYCSRNYYGS	21/37 (57%)	19/42 (45%)	<0.05
VH (87-119)	H9-YCSRNYYGSTYDYWG	25/37 (66%)	28/42 (67%)	<0.05
CH1 (aa 177-207)	H10-SSSLGTQTYICNVHN	10/37 (27%)	12/42 (29%)	<0.1
CH2 (aa 268-300)	H11-KDTLMISRTPEVTCV	12/37 (32%)	14/22 (33%)	<0.08
CH3 (aa 306-336)	H12-NAKTKPREEQYNSTY	11/37 (30%)	10/42 (24%)	<0.05
VL (aa 1-39)	L1-LLTQSPAILSVSPGE	23/37 (62%)	20/42 (48%)	<0.01
VL (aa 1-39)	L2-PGERVSFSCRASQFV	29/37 (78%)	31/42 (74%)	<0.01
VL (aa 1-39)	L3-QFVGSSIHWYQQRTN	19/37 (51%)	22/42 (52%)	<0.01
VL (aa 17-63)	L4-YQQRTNGSPRRTNGS	33/37 (89%)	30/42 (71%)	<0.01
VL (aa 17-63)	L5-SPRLLIKYASESMSG	20/37 (54%)	22/42 (52%)	<0.001
VL (aa 50-97)	L6-MSGIPSRFSGSGSGT	24/37 (65%)	26/42 (62%)	<0.01
VL (aa 50-97)	L7-SGSGSGTDFTLSINT	20/37 (54%)	22/42 (52%)	<0.001
VL (aa 50-97)	L8-SINTVESEDIADYYC	31/37 (84%)	33/42 (79%)	<0.001
VL (aa 79-107)	L9-YCQQSHSWPFTFGSG	28/37 (76%)	33/42 (79%)	<0.01

ADA, anti-drug antibody; CH, constant heavy region; IBD, inflammatory bowel disease; VH, variable heavy-chain region; VL, variable light-chain region.

and H9, 66% and 67% for infliximab and CT-P13 respectively). The epitope H8 were recognised by 57% and 45% of CT-P13 and infliximab patient sera respectively. These results seem to indicate that the prevalent epitope in Infliximab HC of infliximab originator and CT-P13 are localised predominantly in the CDR3 region (Table 4). Small differences in the mean values of peptide recognition between anti-CT-P13 and anti-infliximab sera were not significant to categorise them as an independent peptide. The peptide H10 in the constant region of infliximab was recognised by both sera with higher binding, indicating a dominant epitope in a small number of patients (27% vs 29%, respectively, for infliximab and CT-P13). The sequence of peptide H12 in the Fc domain encompasses the asparagine (Asn)300, which is the only glycosylation site in the infliximab sequence. The predominance of patient sera reactivity to this epitope was rather low but comparable between CT-P13 and infliximab (24% and 30% respectively). These results confirmed the phage ELISA data, indicating that infliximab-treated patients may elicit ADA against therapeutic antibody constant regions. In an alternative representation by heat-map graph filled with interpolation of data, we can observe that both anti-CT-P13 and anti-infliximab sera showed higher binding recognition to H2, H7, H8 and H9 infliximab peptides (Figures S7 and S8). Results from anti-CT-P13 to anti-infliximab sera against infliximab LC peptides demonstrated that the majority of patients have stronger binding to L2, L3, L6 and L9 peptides, which comprised regions between amino acids 1-39 and 50-99 encoding part of the TNF paratope (CDR1 and CDR3) (Figure 5B). The prevalence of IBD patient sera reactivity to these epitopes was higher than to heavy-chain epitopes, with the L3 peptide having the lowest prevalence of sera binding. Analysis of infliximab and CT-P13 antibody reactivity showed comparative binding of IBD patient sera for both infliximab antibodies (L2, 78% vs 74%; L3, 51% vs 52%; L6, 65% vs 62%; L9, 76% vs 79% for infliximab and CT-P13 respectively). The similar immunoreactivity of anti-CT-P13 and anti-infliximab patient sera against these peptides confirmed what we obtained in the phage ELISA experiments. As shown above, a comparison by the heat-map graph of data from anti-CT-P13 to anti-infliximab sera showed higher binding recognition to four LC epitopes with a stronger preference for L2 and L9 that encompasses amino acids 15 to 30 and 90 to 105 (Figures S9 and S10). The collective data shown above indicate that CT-P13 and infliximab have similar immunodominant epitopes, indicating that ADAs against both versions of infliximab recognise similar regions of the molecule.

3.4 | Comparison of infliximab and CT-P13 antigenicity by naïve monoclonal antibodies

The results described above characterised the polyclonal response to infliximab by anti-CT-P13 and anti-infliximab IBD

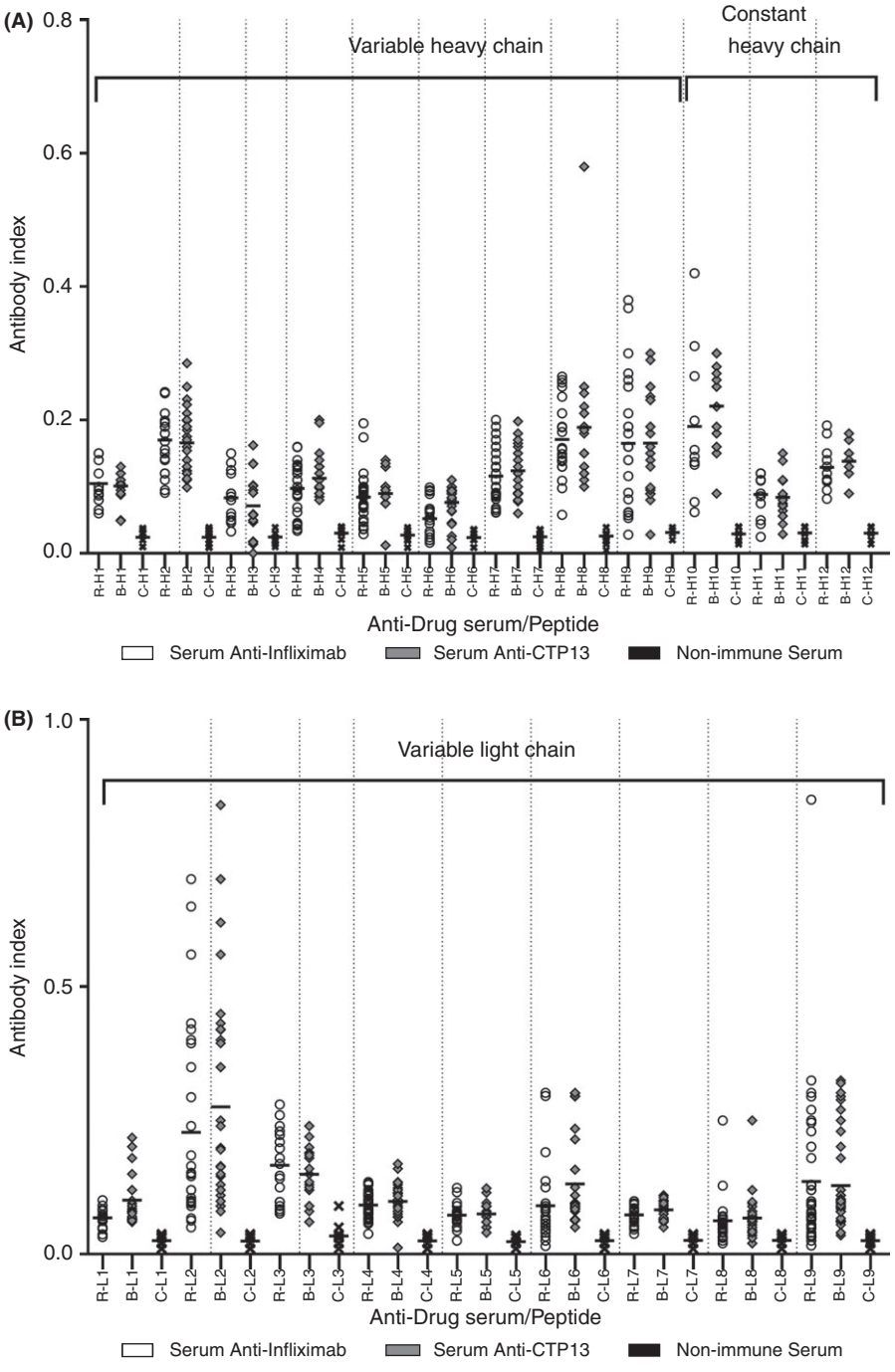

FIGURE 5 Synthetic peptide ELISAs with patient and control sera. (Panel A) Patient and control sera were analysed for antibody binding to synthetic peptides of infliximab heavy-chain in an ELISA format, as detailed in Methods section. (Panel B) Patient and control sera were analysed for antibody binding to synthetic peptides of infliximab Light-chain in an ELISA format, as detailed in Methods section. The antibody indices are shown for patients treated with CT-P13 (B) (n = 42) and infliximab (R) (n = 37) and for control (C) (n = 20) non-immune serum samples analysed in ELISA experiments against synthetic infliximab peptides described in Table 4. Antibody index is calculated by the elimination of background sera binding. The localisation of peptides in infliximab structure was obtained from the ImMunoGeneTics information system (IMGT) and the Crystal Structure Of TNF-alpha In Complex With Infliximab Fab (PDB: 4G3Y_H)

patient sera. To answer the question whether polyclonal antibodies might not detect differences in epitope recognition between CT-P13 and infliximab, we sought to develop synthetic mAbs against CT-P13 and assess whether cross-reactivity was similar to those against infliximab. Using a naïve human Fab library, we submitted the library to three cycles of phage-display against CT-P13. We selected phages encoding Fabs that recognising CT-P13 in ELISA, essentially as described elsewhere.[23] An ELISA, using CT-P13 as a solid phase antigen, was used to determine the relative infliximab-binding capacity of the selected Fab against CT-P13. Twenty-one Fabs were selected in this assay, expressed in *E. coli* BL21 and purified by His-Select Nickel Affinity Gel chromatography.[24] We evaluated the capacity of cross-reactivity for each purified synthetic Fab used at the same concentration to bind Fc and Fab infliximab regions. We prepared Fc and Fab regions of CT-P13 and infliximab by papain digestion on immobilised agarose resin and purification by affinity chromatography. We determined the ratio of anti-infliximab binding versus all five batches of CT-P13 to assess whether different batches may influence antibody reactivity. The ratios of naïve antibody binding between batches of infliximab and CT-P13 were similar to the ratios between batches of CT-P13 themselves (Figure 6B, C). Likewise, when using naïve synthetic antibodies with reactivity against the infliximab Fab domain, a similar ratio of antibody binding

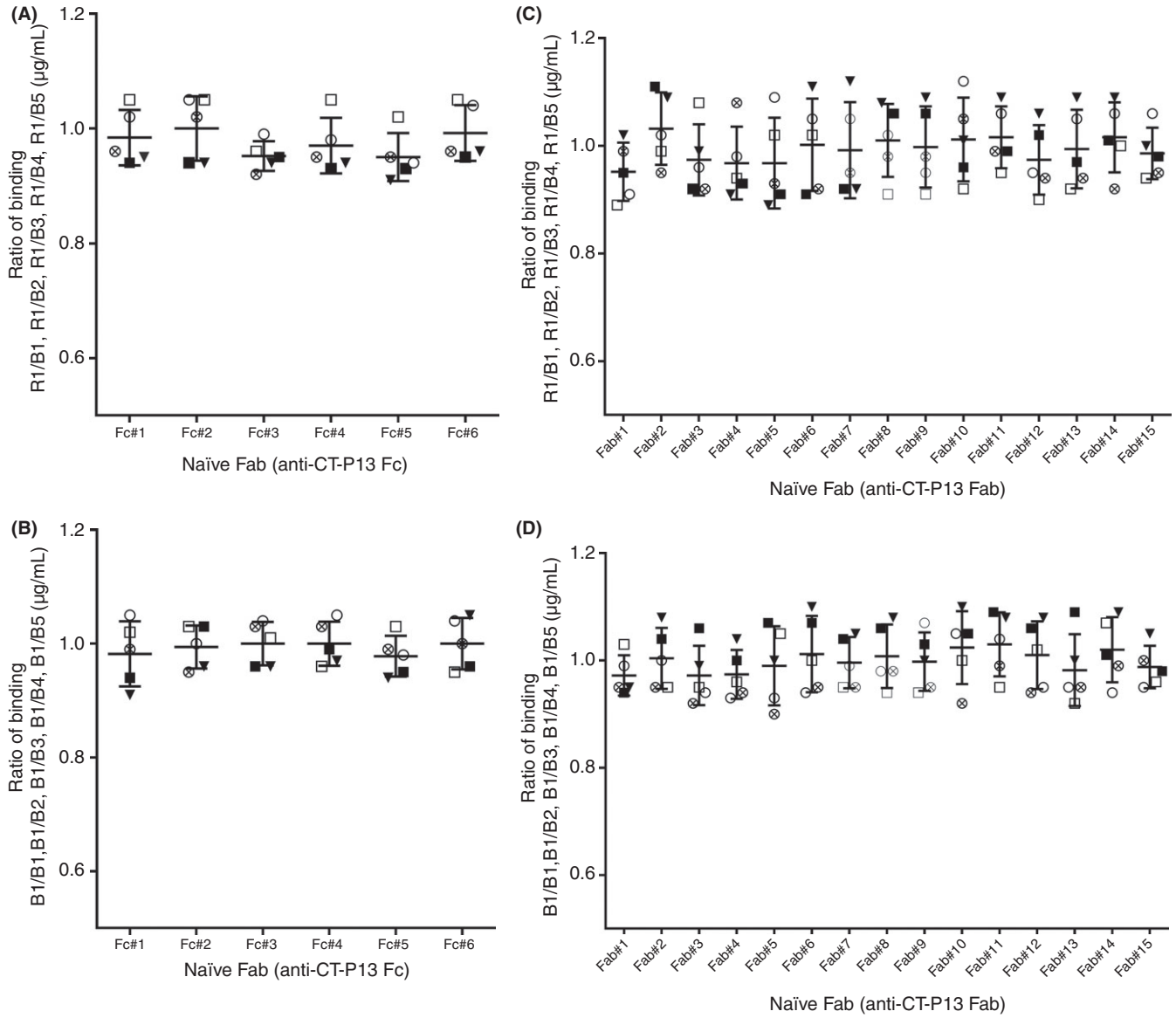

FIGURE 6 (Panel A) Binding to batch R1 of infliximab and five batches of CT-P13 (B1-B5) by six synthetic human Fab against Fc domain of CT-P13. The similar ratio of binding between batch R1 of infliximab and each batch of CT-P13 is shown for every synthetic antibody. The mean of ratios and confidence intervals for each group of batches are shown in all panels. (Panel B) The binding to one batch of CT-P13 (B1) and five batches of CT-P13 (B1-B5) by six synthetic human Fab against Fc domain of CT-P13. The similar ratio of binding between a B1 batch of CT-P13 and all batches of CT-P13 (B1-B5) is shown. (Panel C) The binding to batch R1 of infliximab and five batches of CT-P13 (B1-B5) by fifteen synthetic human Fab against Fab domain of CT-P13. The similar ratio of binding between infliximab and every batch of CT-P13 is shown. (Panel D) The binding to one batch of CT-P13 (B1) and five batches of CT-P13 (B1-B5) by fifteen synthetic human Fab against Fab domain of CT-P13. The similar ratio of binding between a B1 batch of CT-P13 and all batches of CT-P13 (B1-B5) is shown

was observed between infliximab/CT-P13 and CT-P13/CT-P13 (Figure 6D,E). Although we exemplified these data with one batch of infliximab, we obtained similar data with other four batches of this drug (data not shown). The selection of naïve antibodies against CT-P13 and infliximab using phage-display has the advantage to detect epitopes in infliximab that in principle are not detected in IBD patients due to peripheral immune tolerance. Hence, these antibodies appear to indicate that the inter-variability in infliximab structural conformation in various batches of infliximab originator and CT-P13 does not differ more than the inherent intra-variability in CT-P13 or infliximab batches.

3.5 | Comparative antigenicity of infliximab and CT-P13 by IBD-derived monoclonal antibodies

To assess whether monoclonal anti-drug antibodies derived from CT-P13-treated patients have a similar capacity to bind and cross-react with both versions of infliximab, we sought to isolate human Fab domains from these patients. We started by the construction of human Fab libraries derived from three IBD patients with high concentration of anti-CT-P13 antibodies (> 20 µg/mL). Total RNAs were isolated from lymphocytes of human peripheral venous blood. cDNAs coding for immunoglobulin HC and LC were amplified by a

variety of primer combinations designed to amplify a majority of the known human antibody sequences, as described in Methods section. Phage screening was performed using three rounds of panning with the successive removal of low affinity and nonspecifically bound phages. After the final round of panning, 250 mAbs were tested by ELISA against CT-P13. Twelve positive clones that met the criteria (absorbance (*A*) 450 nm >0.5, and the ratio of *A*450 nm for positive control to *A*450 nm for negative control >3) were selected. After purification by affinity chromatography, we used similar concentrations of Fab domains to map the reactivity to infliximab and tested the binding to CT-P13 and cross-reactivity to infliximab. We obtained two anti-CT-P13 mAbs that bound the Fc region of infliximab and ten antibodies that recognised the Fab domain. The Ab index was obtained by elimination of background to human serum. The small number of human antibodies that bound infliximab Fc region was expected due to tolerance effects, however, this result confirmed the peptide mapping data with polyclonal anti-CT-P13 and anti-infliximab. As observed in Figure 7A, the mAB1 Fab domain bound similarly to five different batches of infliximab (0.598 ± 0.035, n = 5) and CT-P13 (0.566 ± 0.032, n = 5) and were statistically equivalent (d = 0.032, Cohen's d = 0.905, df = 8, P = 0.001). This was also true for the mAb2 Fab domain: infliximab (0.392 ± 0.059, df = 5) and CT-P13 (0.398 ± 0.018, df = 5) equivalence (d = 0.006, Cohen's d = 0.131, df = 8, P = 0.033). No systematic effect (ie, always larger or lower Ab index) for a product was found. The intra-product variability (ie, the variability among five batches of each product for each of the two antibodies) was smaller for the CT-P13, s = 0.051 and 0.027 for infliximab and CT-P13 respectively (F test, P = 0.049, df_1 = 8, df_2 = 8). Similar results were obtained for IBD patient mAbs that bound Fab region of infliximab. We tested ten mAbs and compared the Ab index between five different batches of CT-P13 and infliximab (Figure 7B). The intra-product variability (ie, the variability between the five batches of each product for each of the ten Ab) was similar, s_{pooled} = 0.043 and 0.041 for infliximab and CT-P13 respectively (F test, P = 0.43, df_1 = 40, df_2 = 40). Differences in the binding of mAbs to infliximab drugs were found but were expected due to variable affinities of the antibodies for the antigen. No systematic effect for a product

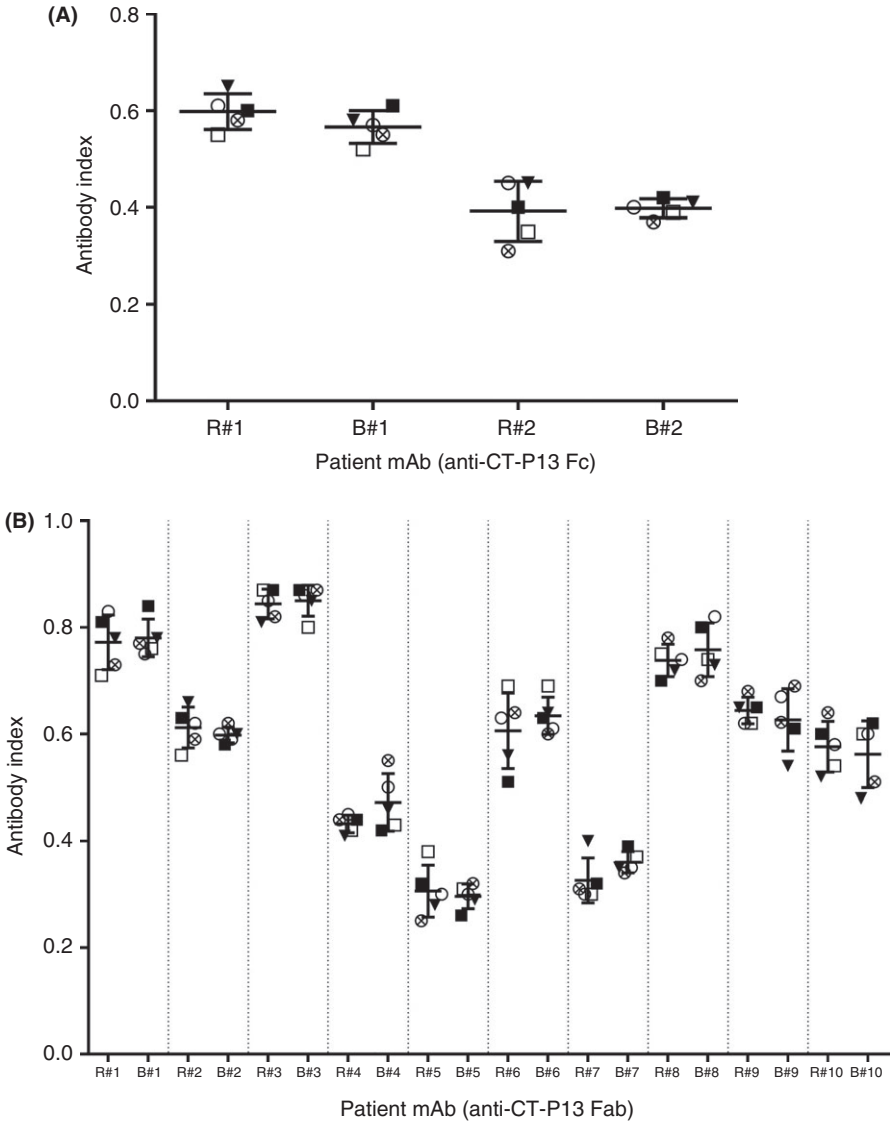

FIGURE 7 (Panel A) Relative binding of two patient monoclonal anti-CT-P13 Fab domains against five batches of CT-P13 and infliximab. The anti-CT-P13 Fab domains were selected by binding to Fc domain of infliximab. The mean of binding and confidence intervals for each group of batches are shown as antibody indices. The similar binding to infliximab and CT-P13 by each anti-CT-P13 mAb is depicted in the graph for every batch analysed. (Panel B) The relative binding of 10 patient monoclonal anti-CT-P13 Fab domains against five batches of CT-P13 and infliximab. The anti-CT-P13 Fab domains were selected by binding to Fab domain of infliximab. The mean of binding and confidence intervals for each group of batches are shown as antibody indices. The similar binding to infliximab and CT-P13 by each anti-CT-P13 mAb derived from IBD patients is depicted in the graph for every batch analysed

(infliximab and CT-P13) was found. When comparing the binding for the various antibodies, using multiple TOST's, statistical non-equivalence between infliximab and CT-P13 was found for mAb4 ($d = 0.04$, Cohen's d = 1.010, $df = 8$, $P = 0.051$) and mAb7 ($d = 0.034$, Cohen's d = 1.030, $df = 8$, $P = 0.087$). For the remaining 8 mAbs, a significant statistical equivalence was found. However, these differences were very small and may be related to the intra-assay variability. These results indicated that the immunogenic response of IBD patients to various versions of infliximab drugs originating from different manufacturing processes was similar. Moreover, we did not detect the presence of new antigenic epitopes in infliximab biosimilar compared with the reference infliximab indicating a similar antigenic response against these two drugs.

4 | DISCUSSION

Biological therapies are inherently variable, creating unavoidable biophysical differences between batches of the same product and its biosimilars while clinical differences (efficacy/safety) remain undetectable. However, there is an unanswered question whether these variations may trigger the development of different antigenic or immunogenic responses compared to the reference drug.[22,25] Thus immunogenicity caused by a switch between a biosimilar and its reference product was hypothesised.[25,26] Nevertheless, a biosimilar is not a new drug in the sense that the primary sequence is the same and the mechanisms of action are similar to the original drug.[1]

During drug switch, immunogenicity may be, in principle, caused by two mechanisms[27,28]: (1) First, the immune system may react to a structural difference between the biosimilar and reference antibody. Such a reaction is highly unlikely with licensed biosimilars since the products have been shown to have comparable stability and immunogenicity in pre-licensing pre-clinical and clinical studies[1,29], (2) The other possibility is that the reference product has a residual immunogenic response and that the immunoglobulin class or specificity will change upon the switch to a biosimilar. In both cases, the activation of T-lymphocytes is required. Thus, activation of T-lymphocytes would require recognition of new linear peptide epitopes in the biosimilar. This argument is also unlikely since the active substance of the CT-P13 has the same amino acid sequence and a similar post-translational profile as compared to its reference product.[1]

To date, there are limited published data regarding the immunogenicity characterisation of CT-P13 in IBD, particularly in the case of switching long-term patients from originator to biosimilar infliximab.[30,31] The immunogenicity against infliximab originator and other infliximab biosimilars is, therefore, an important issue for IBD patients.[7,13,32,33] Thus, it is important to assess whether these potential risks might resist scientific assessment.[28] If the epitopes are similar and no new epitopes are being exposed to biosimilar antibodies or presented in IBD patients, the concerns about switching will be less dependent on the drug and more on the immune status of the patient. Our findings indicate that sera from IBD patients who developed anti-CT-P13 antibodies are cross-reactive with infliximab. Moreover, the cross-reactivity shows that the concentration of anti-drug antibodies capable of recognising both drugs is very similar to the five batches used in this study. Interestingly, we observed no difference in the concentration of IgG4 anti-drug antibodies whether the patients were anti-TNF naïve or infliximab-experienced patients. Therefore, no specific long-term immunity was developed to infliximab and exacerbated to CT-P13 after the switch.[34]

Although the number of patients is limited (n = 16), infliximab-experienced patients treated with CT-P13 did not show significant differences in ADA sera against the two drugs suggesting that tolerance to infliximab epitopes has not occurred. Altogether these results further suggest a similar epitope binding profile between CT-P13 and infliximab sera and that cross-reactivity is not affected by the inherent variability in drug batches.

Similar results were provided before by Ben-Horin et al, showing that anti-infliximab antibodies in patients with IBD recognise and functionally inhibit CT-P13 to a similar degree.[13] We have shown in this study that the opposite is also true, that infliximab CT-P13 does not show the presence of different epitopes compared with infliximab. These results combined, seem to show that both switching and reverse-switching will not elicit different anti-drug antibodies. We identified the most prominent antigenic regions of CT-P13 and infliximab using ADA-positive sera against protein regions and peptide sequences of infliximab. This information is essential to assess whether new immunogenic epitopes might exist in CT-P13 compared to infliximab or whether patients treated with CT-P13 could be more immunogenic to specific peptide regions in infliximab. Our results have shown that anti-infliximab antibody-binding sites were not concentrated in specific regions of the molecule, but they mapped to several amino acid regions between 3-119 in the HC and between amino acids 1-107 in the LC (Table 3).

Our immune-screening study identified three epitopes encoded in the heavy-chain constant region (CH1, CH2 and CH3). Two of these epitopes are located in the human Fc region (H11 and H12) which is confusing due to immune tolerance against these human protein sequences. However, IBD is an immune-mediated disease where immunoglobulin allotypes and inflammatory processes may trigger the development of anti-Fc antibodies of auto-immune nature. We have observed such cases with the development of rheumatoid factor or even reports showing the development of antibodies against constant regions of infliximab. The H12 peptide epitope incorporates the glycosylation site of infliximab at the asparagine amino acid 300, and the H11 peptide sequence is just upstream of this sequence. In our assay, the binding to H11 and H12 was performed in conditions without peptide glycosylation. However, it is also conceivable that due to native glycosylation of infliximab, these antibodies were raised but have difficulty to bind to the Fc region. Nevertheless, both CT-P13 and infliximab sera recognised H11 and H12 epitope peptides similarly, suggesting that putative differences in glycosylation between the two versions of infliximab did not affect antigenicity.[1] We have confirmed this result by comparing

reactivity of anti-CT-P13 and anti-infliximab sera against deglycosylated infliximab and CT-P13 (data not shown).

Overall, our findings are in agreement with earlier studies that have suggested that antigenic epitopes for infliximab localise to the paratope region of the antibody.[35] Predominantly, these epitopes are confined in the CDR1 and CDR3 of infliximab HC, in the LC at the CDR3 and close to the CDR1. These complementary determining regions are strongly involved in the high-affinity interaction with TNF and responsible for its neutralisation. This observation is in agreement with the decline of infliximab trough concentration in sera and the neutralisation of its clinical activity in these patients. What is interesting to note from Figure 5A,B (also Figures S7 and S10), is that the antigenic response against the paratope of CT-P13 and infliximab is strikingly similar. Despite the flexible nature of antibody CDR regions, both antibodies seem to show very similar immunoreactivities to anti-drug antibodies. Results also showed that the frequency of patients with reactivity against peptides H2, H7, H8, and L2, L3 and L9 is very similar between anti-CT-P13 and anti-infliximab antibodies. These epitopes are of interest for future assessment in the clinic as biomarkers since the majority of patients show detectable antibodies against these sequences. In our assay, we did not identify a specific epitope peptide recognised by the totality of IBD sera in this study. These results suggest heterogeneity of epitopes presented to the immune system which probably depends on the HLA background of patients.

The immunoreactivity of anti-CT-P13 and anti-infliximab originato sera against the light-chain peptides are greater than to heavy-chain. It is highly speculative, but this result can be explored regarding IBD patient HLA specific presentation of infliximab LC peptides. The presence of HLA epitopes present in the LC or HC infliximab peptides may provide the clues about the role of IBD patient HLA to develop anti-infliximab immunogenicity. Similarly to a previous study on infliximab epitopes, we have detected strong sera reactivity of both anti-CT-P13 and anti-infliximab sera to the vicinity of the LC CDR2 region.[35] The frequency of patients that respond to this epitope is very high, but with great variability in the sera titre. Although the binding is not direct to the CDR region, the antibody recognition might induce steric hindrance and neutralise TNF ligation. Although we used only three patients, the anti-CT-P13 mAbs recognised similarly different batches of original and biosimilar infliximab. It is plausible to conclude that putative differences in conformation between the 2 drugs do not raise biosimilar-specific high-affinity antibodies, and therefore, develop similar neutralising antibodies against both versions of infliximab. A limitation of this study should be acknowledged since we have not raised fully human IgG from these libraries but a selection of combinatorial LC/HC pairs. However, the main objective of this study was to compare the antigenic differences between CT-P13 and infliximab, and with this strategy, we can derive anti-infliximab antibodies independent of the immune status of the host. When taken together, these data strongly support a highly similar antigenic profile for infliximab and CT-P13, probably due to common immunodominant epitopes on these two infliximab versions. The consequent clinical implications are that switching between infliximab/CT-P13 will not raise new immunogenic/antigenic reactions. Furthermore, these data indicate safe switching between the two drugs in ADA-negative patients due to the low risk for evoking immunogenicity. This view is supported by recent clinical studies showing that switches between infliximab and CT-P13 do not show differences in immunogenicity and safety.[23,24,31,36] Besides the benefit for patients by facilitating easier and earlier access to biologic treatments through reduced drug costs, biosimilars can also bring innovation to targeted treatments. Although we can conclude from this work about safe switching, interchangeability was not directly addressed here. Interchangeability represents another level of complexity since multiple switches may, theoretically, promote immunogenicity through minor epitopes sensitisation by repeated exposures which may evade detection by the present methodology. While this study addresses comparative immunogenicity in a controlled manner, we cannot also conclude about nonmedical switching since there are other aspects which may influence the outcomes of nonmedical switch which lie outside this study. The assessment of immunogenicity to a biologic therapy is a key element of innovation to sustain clinical efficacy of biosimilar therapies. The evolution of ADA appearance, the kinetics of epitope detection, and the biosimilar epitope dominance between different biosimilars may help to avoid or predict the development of therapeutic secondary failures in IBD patients. Because of these implications, future studies should answer these questions to advance further the field of biosimilar therapies in IBD.

To summarise, this study is the first to compare specific antigenic epitopes between CT-P13 and infliximab originator, and identify antibody-binding sites on different versions of infliximab with polyclonal and monoclonal sera. The results strongly support a similar antigenic profile for infliximab and CT-P13. The consequent clinical implication is that switching between infliximab/CT-P13 will likely raise no new immunogenic/antigenic reactions. These data point toward a safe switching between the two drugs in ADA-negative patients.

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Author contributions: MS, RA, II, PB, ACCS, AB, JG, IB, MY performed the research; LG, FAS, collected, and analysed data; AA, MC, MC contributed to the design of the study; JDA, JC, TD, JEF, CP, JT, CLV, DT, GF, AS, IR, LC, FM, GH, SB, PLL, SD designed the research study and wrote the paper. All authors approved the final version of the manuscript.

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LINKED CONTENT

This article is linked to Pouillon et al and Goncalves et al papers. To view these articles visit https://doi.org/10.1111/apt.14847 and https://doi.org/10.1111/apt.14860.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section at the end of the article.

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APPENDIX 1

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