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# Serum Neutrophil Biomarkers to Predict Crohn's Disease Progression and Infliximab Treatment Outcomes

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## ABSTRACT

**Background and aims:** Predicting the treatment outcomes of biological therapies is an unmet need in Crohn's Disease. In this study, we explored the potential of serum neutrophil-related biomarkers to predict infliximab therapeutic results and disease progression in Crohn's Disease patients, over a 2-year period, in a real-world setting.

**Methods:** The study included 100 asymptomatic Crohn's Disease patients in the IFX maintenance phase from the prospective, observational, multicenter DIRECT study. Patients were categorized according to a composite outcome reflecting progression that included surgery, hospitalizations, new fistulae, abscess or stricture, and drug treatment escalation. Serum neutrophil elastase, lipocalin-2, lactoferrin, and resistin (non-neutrophil control) were analyzed via multiplex magnetic bead assays at multiple touchpoints. Fecal calprotectin was assessed by ELISA.

**Results:** Over up to 2 years of follow-up, serum biomarkers did not differentiate between the composite outcome groups, whereas fecal calprotectin was significantly higher in patients with worse outcomes. During the infliximab maintenance phase, there was a significant, sustained reduction of neutrophil elastase ($p < 0.001$), lipocalin-2 ($p < 0.001$), and lactoferrin ($p < 0.001$), but not of resistin, despite stable neutrophil levels. Correlations between NE and NGAL levels were strong (Pearson correlations 0.75–0.85); all other correlations were of small magnitude.

For a complete listing of the GEDII, see the Acknowledgments section.

**Conclusion:** Our real-world data do not support using serum neutrophil elastase, lipocalin-2, or lactoferrin concentrations as predictors of treatment outcomes or disease evolution in infliximab -treated Crohn's Disease patients. On the other hand, the sustained decrease in biomarkers over time suggests that neutrophil stabilization might be an additional infliximab mechanism of action.

## 1 | Introduction

The anti-TNFα antibody infliximab (IFX) revolutionized the management of chronic inflammatory bowel diseases (IBD) [1]. Over time, the knowledge on the mechanism of action of IFX evolved from the awareness of a simple neutralization of soluble- and membrane-TNF to pleiotropic effects, including the promotion of T-cell apoptosis, induction of M2 macrophages, and activation of complement-dependent and antibody-dependent cytotoxicity [2, 3]. Despite these advances, 10%–30% of patients do not respond to IFX or other biologics [4]. Predicting the outcomes of these therapies is therefore a critical need to prevent unnecessary exposure to adverse effects and increase treatment efficacy [4].

Though ill-studied, neutrophils are a promising source of potentially reliable markers [4]. The induction of disease remission by IFX has been reported to be dependent on the rapid block of neutrophil recruitment [5]. However, unlike for macrophages and T-cells, the direct interplay between IFX and neutrophils has seldom been explored [3, 5].

The STRIDE-II recommendations consider that the normalization of the neutrophilic fecal calprotectin (FC) is, along with serum C-reactive protein (CRP) and erythrocyte sedimentation rate, a short-term treat-to-target strategy, attesting the utility of analyzing granulocytes [6]. Other neutrophil-related fecal compounds have been studied in IBD [7], but the cumbersome collection procedure is a common barrier to their more widespread use [8, 9]. While resorting to blood-based biomarkers could overcome this flaw [8], interest has been low. A recent systematic review summarized data on the ability of blood-based neutrophil-related biomarkers to predict the evolution of IBD [10]. Encouraging results were found for nCD64, TREM1 (triggering receptor expressed on myeloid cells 1), and CPa9-HNE (neutrophil elastase-generated calprotectin fragment), but data were too fragmented to draw definite conclusions. Remarkably, even though antibacterial lactoferrin (LAC) is a well-known fecal biomarker in IBD [11], no study was captured in the referred systematic review. The same applied to serum neutrophil elastase-2 (NE), an anti-inflammatory proteinase from the primary neutrophilic granules [7, 12]. Only a single small study involved neutrophil gelatinase-associated lipocalin (NGAL; lipocalin-2), a NET-associated bacteriostatic protein from the specific granules [13].

This analysis explored the potential of serum biomarkers associated with neutrophil granules (NE, NGAL, and LAC) to predict IFX outcomes and disease progression in CD patients.

## 2 | Methods

### 2.1 | Selection of Patients

DIRECT ("*Study to investigate the correlation of fecal calprotectin with serum Drug levels and development of an antI-dRug antibodiEs among adult patients with inflammatory bowel disease reCeiving anti-TNF-alfa treatment or Vedoluzimab treatment*") was a multicenter, prospective, real-world study, including adult patients diagnosed with Crohn's disease (CD) or ulcerative colitis (UC), carried out in eight Portuguese IBD specialized centers, between May 2016 and October 2019. Study methods are described in detail elsewhere [14]. Briefly, patients were followed up for up to 2 years; blood samples were collected according to routine clinical care and stored at −80°C until analysis by a central laboratory. In each visit, patients were instructed to report their health status (abdominal pain and soft/liquid stool frequency) by filling a questionnaire (patient-reported outcome 2 [PRO-2]) regarding the week before each infusion [15].

For this analysis, two DIRECT subpopulations were selected. Both groups 1 and 2 included patients cumulatively (i) diagnosed with CD, (ii) asymptomatic throughout the follow-up period, (iii) receiving IFX maintenance treatment for ≥ 14 weeks, and (iv) with serum samples collected at baseline (V1), 12 months (± 6 weeks; V2), and 24 months (± 8 weeks, V3). Clinical activity was defined by abdominal pain score > 1 or liquid-to-very-soft stool frequency > 1.5 in PRO-2 in two consecutive or at least three non-consecutive visits during the follow-up period. All patients were asymptomatic according to this definition across the entire follow-up period. Higher PRO-2 scores reported in one or two non-consecutive visits were dubbed "isolated symptomatology". Group 1 included 50 patients who achieved the composite outcome during the follow-up period. Group 2 ($n$ = 50) patients were age- and gender-matched to Group 1 but did not reach the composite outcome. Matching was performed using the SPSS 28.0.1 function Case-Control Matching with gender tolerance = 0 and age tolerance = 5 years. The best matched 50 pairs were selected.

No formal sample size was calculated in this exploratory study. All patients voluntarily signed a written informed consent. The study was approved by local ethics committees and the Portuguese Data Protection Authority and conducted according to the Declaration of Helsinki principles.

### 2.2 | Objectives and Endpoints

The primary objective of this analysis was to assess if serum levels of a biomarker panel of NE, NGAL, LAC, and resistin

**Summary**

- Summarize the established knowledge on this subject
  - There are no established blood-based biomarkers to predict infliximab treatment outcomes in Crohn's disease.
  - Normalization of the neutrophil-related fecal calprotectin is a recommended short-term treat-to-target strategy in Crohn's disease.
- What are the significant and/or new findings of this study?
  - Serum levels of neutrophil-related neutrophil elastase, NGAL and lactoferrin were not useful to predict treatment outcomes in infliximab-treated Crohn's disease patients.
  - Neutrophil elastase, NGAL and lactoferrin serum levels significantly decreased over 2 years of infliximab treatment despite stable neutrophil levels.
  - Stabilization of neutrophil degranulation might be a novel mechanism of action of infliximab.

(pro-inflammatory cytokine expressed mainly in peripheral blood mononuclear cells and adipocytes [16] and included as a non-neutrophilic control) could predict disease progression and treatment outcomes. A composite outcome (described by Magro et al. [14]) was defined with two clusters: clinical-related items (first occurrence of at least one of: abdominal surgery; IBD-related hospitalizations; new fistulae, abscess or stricture; or isolated symptomatology) and drug-related items (first occurrence of at least one of: being prescribed or having received at least one course of oral corticosteroids or more than 10 mg of prednisolone daily; *de novo* azathioprine or methotrexate; switch of biological therapy due to clinical unresponsiveness [to adalimumab, golimumab, vedolizumab, or ustekinumab]; increase of azathioprine dose by any reason other than weight fluctuation; and IFX dose escalation or interval reduction). Secondary objectives included describing changes in serum concentrations over the IFX maintenance period and exploring associations between serum levels of biomarkers and CRP, FC, IFX and anti-IFX levels, and disease activity indexes [as recorded by the physician-reported Harvey-Bradshaw Index (HBI) or the patient-reported outcome (PRO)-HBI and PRO-2 at each visit].

### 2.3 | Determination of Serum Levels of Biomarkers

Serum NE, NGAL, LAC, and resistin levels were measured using a magnetic bead-based Milliplex assay kit (Merck Millipore Corporation, MA, USA; HSP3MAG-63K) in a Luminex 200, according to the manufacturer's instructions. This technique uses analyte-specific microspheres ("beads") coated with capture antibodies, which are identified by a unique fluorescent dye. The association between the biotinylated detection antibodies' radiation and the fluorescent radiation from the bead-specific dye enables the precise and simultaneous quantification of multiple compounds. To minimize errors due to inter-assay variability, time between experiments, and order of assays, samples were randomly ascribed to a certain microplate and position, ensuring that each microplate contained a proportional number of V1,V2 and V3 samples of each analyte and groups. Automatically extrapolated values outside the detection range were considered valid, and undefined values outside the detection range were excluded from the analyses. FC was analyzed by ELISA; CRP, IFX, and anti-IFX levels were assessed according to routine practice and analyzed as described elsewhere [14].

### 2.4 | Data Processing and Statistical Analysis

Categorical variables were described through absolute ($n$) and relative (%) frequencies, while continuous variables were described as mean and standard deviation, or median, interquartile (IQR) range, and minimum and maximum, when appropriate. Demographic characteristics were compared by independent t-tests, Chi-square or exact Fisher tests for categorical variables, and by independent t-tests for continuous variables (Table 1). The primary objective (disease progression prediction) was analyzed by Cox regression, which looked at the relationship over time between biomarker serum levels (at V1, V2 and V3), gender, age, FC, IFX, anti-IFX, neutrophil serum levels and CRP, and the achievement of composite outcome (Table 2). Changes over time in the maintenance period (secondary objective) were analyzed by Friedman tests with Dunn's correction for multiple comparisons and listwise deletion. Same timepoint comparisons between V1-V1 and V3-V3 were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. Correlations between serum levels of biomarkers and CRP, FC, IFX and anti-IFX levels, and disease activity indexes (secondary objective) were obtained through Pearson correlations (Table 3). Differences between biomarker concentrations by class of IFX levels (IFX $<$ 3 μg/mL; 3 $\leq$ IFX $\leq$ 7 μg/mL; IFX $>$ 7 μg/mL) were analyzed by one-way ANOVA with Tukey's test for multiple comparisons (Supporting Information S1: Figure S1). $p$-values below 0.05 were considered statistically significant and confidence intervals were set to 95%. Statistical analyses were performed in GraphPad Prism 8.0.2 (MA, USA) and IBM SPSS 28.0.1 (NY, USA).

## 3 | Results

### 3.1 | Patient Disposition and Baseline Characteristics

Out of 332 DIRECT CD patients treated with IFX, 128 were in the maintenance phase, asymptomatic throughout the study, and had serum samples available at baseline, 12 months, and 24 months (Figure 1). Of these, 50 patients who achieved the composite outcome were randomly chosen (Group 1), and 50 age- (tolerance of 4 years) and gender-matched patients were selected for Group 2. Groups 1 and 2 were similar in all characteristics, except for FC at baseline (Table 1).

**TABLE 1** | Demographic characteristics of the population.

| | Direct CD patients in IFX maintenance phase | | | |
|---|---|---|---|---|
| | **Group 1 Composite outcome yes** | **Group 2 Composite outcome no** | ***p-value* 1 versus 2** | **All patients (1 + 2)** |
| *n* | 50 | 50 | — | 100 |
| Age | | | | |
| Mean (sd) | 37.9 (11.5) | 38.3 (12.1) | 0.872[a] | 38.1 (11.7) |
| Median (Q1–Q3) | 36.5 (28.8–45.3) | 35.5 (28.0–47.0) | | 36.0 (28.3–46.0) |
| Male, *n* (%) | 35 (70) | 35 (70) | — | 70 (70) |
| BMI ($kg/m^2$) | | | | |
| Mean (sd) | 23.7 (4.1) | 24.5 (3.5) | 0.319[a] | 24.1 (3.8) |
| Median (Q1–Q3) | 22.8 (20.6–26.3) | 24.2 (22.2–27.2) | | 23.4 (21.0–26.8) |
| Smoking, *n* (%) | | | 0.948[b] | |
| Never smoked | 31 (62) | 31 (62) | | 62 (62) |
| < 10/day | 8 (16) | 6 (12) | | 14 (14) |
| 10–20/day | 3 (6) | 3 (6) | | 6 (6) |
| > 20/day | 0 (0) | 1 (2) | | 1 (1) |
| Ex-smoker | 8 (16) | 9 (18) | | 17 (17) |
| Disease location, *n* (%) | | | 0.706[b] | |
| L1 (terminal ileum) | 15 (30) | 15 (30) | | 30 (30) |
| L1 + L4 | 2 (4) | 5 (10) | | 7 (7) |
| L2 (colon) | 12 (24) | 8 (16) | | 20 (20) |
| L2 + L4 | 0 (0) | 0 (0) | | 0 (0) |
| L3 (ileocolon) | 15 (30) | 15 (30) | | 13 (30) |
| L3 + L4 | 6 (12) | 7 (14) | | 13 (13) |
| Perianal disease, yes, *n* (%) | 17 (34) | 15 (30) | 0.668[b] | 32 (32) |
| Disease classification, *n* (%) | | | 0.319[b] | |
| Fistulae or anal abscesses | 3 (6) | 1 (2) | | 4 (4) |
| Non-stenosing + non-penetrating | 29 (58) | 22 (44) | | 51 (51) |
| Penetrating | 10 (20) | 18 (36) | | 28 (28) |
| Penetrating + stenosing | 2 (4) | 3 (6) | | 5 (5) |
| Stenosing | 6 (12) | 6 (12) | | 12 (12) |
| Corticodependent, *n* (%) | 23 (46) | 17 (34) | 0.221[b] | 40 (40) |
| Corticoresistant, *n* (%) | 1 (2) | 3 (6) | 0.617[c] | 4 (4) |
| IFX treatment duration before baseline (years) | | | | |
| Mean (sd) | 3.7 (3.4) | 4.5 (3.7) | 0.238[a] | 4.1 (3.6) |
| Median (Q1–Q3) | 3.0 (1.0–6.0) | 3.5 (1.0–7.0) | | 3.0 (1.0–6.0) |
| Therapy at baseline | | | 0.749 † | |
| Monotherapy | 28 (56) | 29 (58) | | 57 (57) |
| Combined therapy | 22 (44) | 20 (40) | | 42 (42) |
| FC Baseline (μg/g) | | | **0.032**[a] | |
| Mean (sd) | 667.5 (1480.1) | 181.5 (189.7) | | 427.2 (1082.8) |
| Median (Q1–Q3) | 237.5 (95.3–610.3) | 87.0 (55.0–300.0) | | 122.0 (60.0–426.0) |
| CRP plasma levels at baseline (mg/L) | | | 0.392[a] | |
| Mean (sd) | 5.5 (8.9) | 4.2 (6.4) | | 4.9 (7.7) |
| Median (Q1–Q3) | 2.7 (1.7–9.6) | 1.6 (0.8–4.6) | | 2.0 (0.9–5.7) |

(Continues)

**TABLE 1** | (Continued)

| | Direct CD patients in IFX maintenance phase | | | |
|---|---|---|---|---|
| | **Group 1 Composite outcome yes** | **Group 2 Composite outcome no** | ***p-value* 1 versus 2** | **All patients (1 + 2)** |
| IFX serum levels at baseline (μg/mL) | | | 0.635[a] | |
| Mean (sd) | 7.9 (11.1) | 8.9 (9.1) | | 8.4 (10.2) |
| Median (Q1–Q3) | 4.6 (1.6–9.6) | 6.6 (4.6–9.7) | | 5.7 (3.3–9.6) |
| Anti-IFX serum levels at baseline (μg/mL) | | | 0.269[a] | |
| Mean (sd) | 0.7 (0.8) | 0.6 (0.4) | | 0.7 (0.6) |
| Median (Q1–Q3) | 0.5 (0.3–0.9) | 0.5 (0.3–0.7) | | 0.5 (0.3–0.8) |

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FC, fecal calprotectin; IFX, infliximab; sd, standard deviation.
[a]Independent *t*-test.
[b]Chi-square.
[c]Exact Fisher Test.

**TABLE 2** | Cox regression at baseline (V1) and 1-year (V2) explaining the future achievement of composite outcome.

| | V1 | | V2 | |
|---|---|---|---|---|
| **Factor** | **HR (95% CI)** | ***p*-value** | **HR (95% CI)** | ***p*-value** |
| NE serum levels (ng/mL) | 0.989 (0.701–1.396) | 0.951 | 1.068 (0.585–1.949) | 0.830 |
| NGAL serum levels (ng/mL) | 0.908 (0.670–1.230) | 0.533 | 0.749 (0.383–1.466) | 0.399 |
| LAC serum levels (ng/mL) | 1.000 (1.000–1.001) | 0.083 | 0.996 (0.988–1.005) | 0.383 |
| RES serum levels (ng/mL) | 0.994 (0.984–1.005) | 0.304 | 1.001 (1.000–1.001) | 0.053 |
| Gender | 1.069 (0.754–1.517) | 0.707 | 0.848 (0.542–1.327) | 0.470 |
| Age (years) | 0.993 (0.966–1.022) | 0.651 | 0.983 (0.950–1.016) | 0.303 |
| FC levels (μg/g) | 1.000 (1.000–1.000) | **0.025** | 1.001 (1.000–1.002) | **0.021** |
| IFX serum levels (μg/mL) | 0.994 (0.958–1.032) | 0.755 | 1.001 (0.969–1.033) | 0.975 |
| Anti-IFX serum levels (μg/mL) | 1.349 (0.884–2.059) | 0.165 | 1.117 (0.807–1.545) | 0.504 |
| Neutrophil serum levels ($\times 10^9$/l) | 1.080 (0.881–1.326) | 0.459 | 0.921 (0.682–1.245) | 0.594 |
| CRP serum levels (mg/L) | 1.019 (0.978–1.062) | 0.374 | 1.003 (0.923–1.089) | 0.951 |

Abbreviations: CI, confidence interval; CRP, C-reactive protein; FC, fecal calprotectin; HR, hazard ratio; IFX, infliximab; LAC, lactoferrin; NE, neutrophil elastase; NGAL, lipocalin 2; OR, odds ratio; RES, resistin.

### 3.2 | Evolution of Serum Biomarker Levels During the Maintenance Phase

NE, NGAL, and LAC levels significantly decreased during the IFX maintenance period, even after normalization by neutrophil count (Figure 2A–C). Serum neutrophil levels remained stable over the entire maintenance period (V1 vs. V3: 4.01 $\times$ $10^9$/L vs. 4.08 $\times$ $10^9$/L, $p = 0.760$). No differences were detected between the two outcome groups at any time point. Corticosteroid dependence at study entry (dependents: $n = 40$; non-dependents: $n = 60$) did not influence the levels of biomarkers, except for NGAL, where being corticodependant was associated with significantly higher levels at baseline (Figure 2B). Only three patients increased or started corticosteroids over the 2-year follow-up period. Unlike serum neutrophil-related biomarkers, resistin levels remained stable over the 2-year period of follow-up period (Figure 2D). FC was overall steady, but patients who reached the composite outcome during the study displayed significantly higher FC levels at baseline (Figure 2E).

### 3.3 | Prediction of Treatment Outcomes

The levels of biomarkers at baseline (V1) and after 1 year (V2) of follow-up were not able to predict treatment outcomes over the course of 2 years, as measured by the association with a composite outcome encompassing both drug- and clinical-related items (Figure 2, center-left panel of each biomarker). Sensitivity analysis considering only drug- or clinical-related items did not yield significant discriminative power either (data not shown). To disentangle possible confounders and account for time, Cox regressions were performed to ascertain the effects of NE, NGAL, LAC, and resistin serum levels, FC levels, gender, age, and CRP, IFX, and anti-IFX serum levels on the likelihood of achieving the composite outcome. Higher FC levels at both V1 and V2 were associated with a significantly greater likelihood of progressing to the composite outcome (worse output) (Table 2). No other factor was a significant contributor to the predictive model (Table 2).

**TABLE 3** | Correlation between biomarker panel baseline levels and inflammatory markers, clinical indexes, and infliximab serum levels at baseline (V1) and 2 years/end of follow-up (V3).

| | Baseline (V1) | | | | 2 years/end of follow-up (V3) | | | |
|---|---|---|---|---|---|---|---|---|
| | NE | NGAL | LAC | RES | NE | NGAL | LAC | RES |
| CRP (mg/L) | −0.048 | 0.105 | 0.000 | −0.046 | 0.177 | **0.252** | −0.074 | **0.238** |
| Hemoglobin (g/dL) | 0.164 | 0.077 | 0.111 | 0.095 | −0.008 | −0.034 | −0.053 | −0.102 |
| Ferritin (ng/L) | 0.111 | −0.015 | −0.060 | −0.043 | 0.040 | 0.065 | 0.036 | 0.091 |
| Iron (μg/dL) | 0.082 | 0.014 | −0.077 | 0.035 | −0.021 | −0.129 | −0.035 | −0.057 |
| Neutrophils (x10^9/L) | **0.230** | **0.304** | **0.242** | 0.063 | **0.375** | **0.392** | 0.141 | 0.154 |
| IFX levels (μg/mL) | −0.016 | −0.021 | −0.068 | 0.013 | 0.187 | 0.108 | 0.151 | 0.119 |
| Anti-IFX levels (μg/mL) | −0.167 | −0.135 | −0.045 | −0.136 | −0.044 | 0.015 | 0.000 | 0.153 |
| HBI | −0.071 | 0.047 | 0.030 | 0.087 | 0.000 | 0.030 | 0.084 | −0.049 |
| PRO-HBI | 0.008 | 0.084 | −0.080 | 0.109 | **0.299** | **0.346** | 0.084 | **0.271** |
| CD disease location | 0.157 | 0.152 | −0.016 | 0.065 | −0.008 | −0.028 | −0.048 | 0.007 |
| CD disease classification | −0.161 | −0.155 | **−0.234** | 0.117 | 0.095 | 0.117 | −0.095 | 1.145 |
| NE | — | **0.746** | 0.112 | **0.266** | — | **0.852** | **0.200** | 0.179 |
| NGAL | **0.746** | — | **0.325** | **0.285** | **0.852** | — | **0.248** | **0.210** |
| LAC | 0.112 | **0.325** | — | 0.040 | **0.200** | **0.248** | — | 0.005 |
| RES | **0.266** | **0.285** | 0.040 | — | 0.179 | **0.210** | 0.005 | — |
| FC (ug/g) | 0.133 | **0.206** | 0.011 | −0.073 | 0.187 | 0.137 | −0.040 | 0.014 |

*Note:* Bolded values are significant Pearson correlations at the 0,05 level (2-tailed).
Abbreviations: CD, Crohn's Disease; CI, confidence interval; CRP, C-reactive protein; FC, fecal calprotectin; HBI, Harvey-Bradshaw Index; IFX, infliximab; LAC, lactoferrin; NE, neutrophil elastase; NGAL, lipocalin 2; PRO-HBI, patient reported outcomes Harvey-Bradshaw Index; RES, resistin.

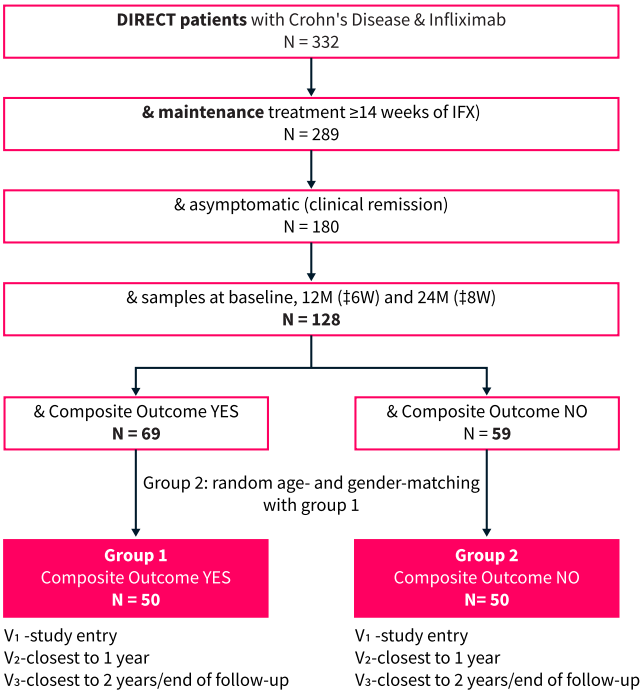


**FIGURE 1** | Patient disposition and blood collection points.

### 3.4 | Correlation With Inflammatory Biomarkers, Clinical Indexes, and Serum Infliximab Levels

Finally, we evaluated the correlation between the biomarker panel and the following parameters at the beginning (V1) and at 2 years of follow-up (V3) (Table 3): plasmatic levels of CRP, hemoglobin, ferritin, iron, neutrophil count, serum IFX and anti-IFX levels, clinical activity (HBI and PRO-HBI), disease location and disease classification. Correlation between biomarkers was also assessed. Apart from the relationship between NE and NGAL at all timepoints, correlations were of small magnitude. At V1, NE, NGAL, and lactoferrin were weakly correlated with neutrophil count; the same was observed at V3 for NE and NGAL. At V3, NE, NGAL, and resistin correlated with PRO-HBI; NGAL and resistin also correlated with CRP. Clustering IFX levels in three classes (IFX < 3 μg/mL; 3 ≤ IFX ≤ 7 μg/mL; IFX > 7 μg/mL) did not change the outlook (Supporting Information S1: Figure 1).

### 3.5 | Effect of Storage Time

Storage time (the time between collection of sample and lab analysis) at −80°C was not significantly correlated with the levels of any biomarker. Indeed, V1 (typically older) samples tended to be associated with higher levels of biomarkers than those collected at V3 (typically more recent).

## 4 | Discussion

The quest for ideal biomarkers in IBD has yielded clear advances over the last decades, from diagnosis to prognosis [17]. However, elusive goals such as predicting the outcomes of the biological therapies and preventing unnecessary exposure of non-responders persist. Neutrophils are a promising source of

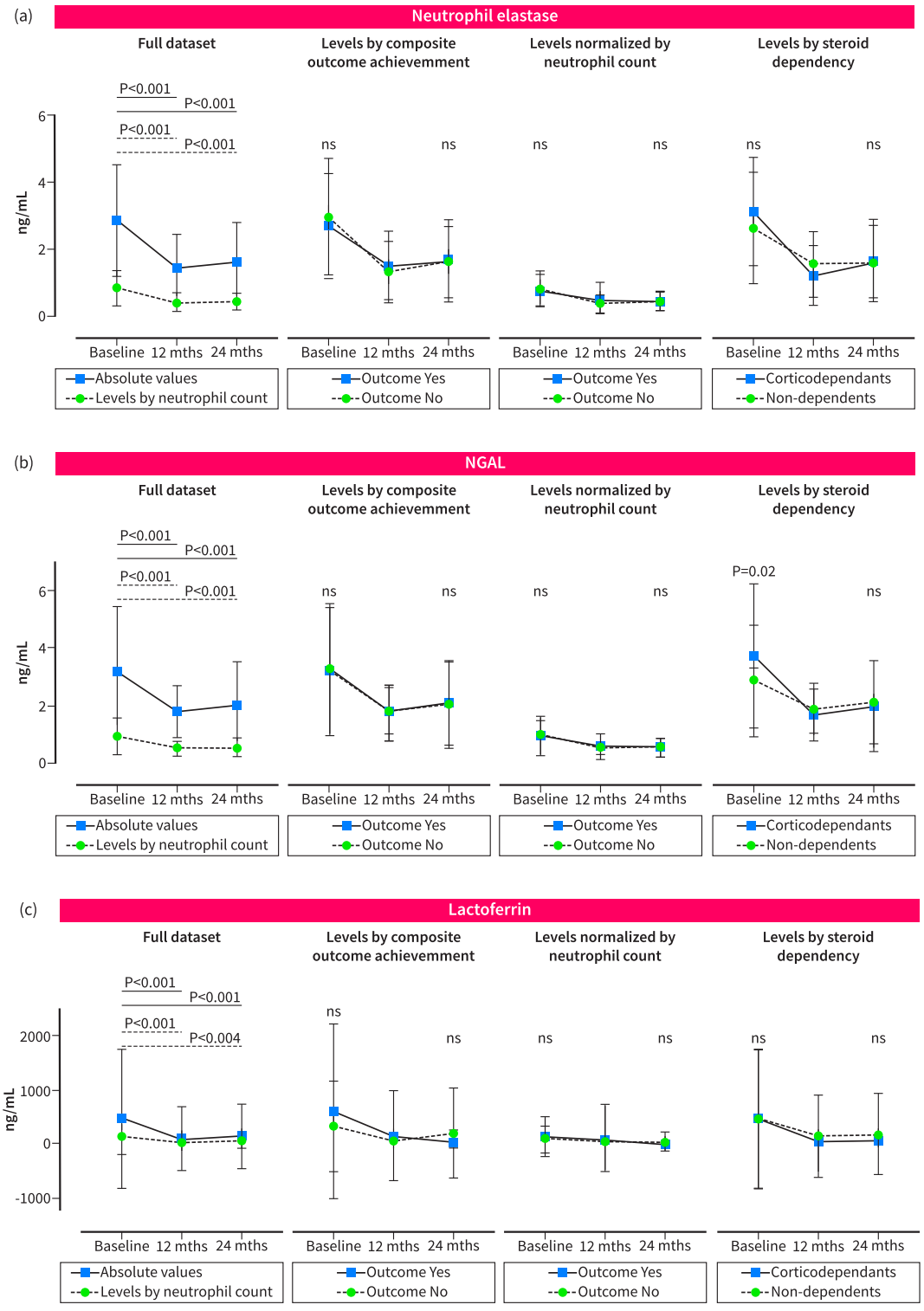


**FIGURE 2** | Changes in serum (ng/mL; panels A–D) and fecal (μg/g; panel E) concentrations of each biomarker over up to 2 years of infliximab maintenance treatment. Error bars: Standard deviation. *p*-values below 0.05 were considered statistically significant.

reliable markers [4]. Indeed, in a recent study evaluating the reliability of histologic indexes to assess CD activity, two neutrophil-based items were included in exploratory colonic, ileal and colonic-ileal indexes, underscoring the importance of neutrophils in driving mucosal damage in CD and as potential therapeutic targets [18].

In this study, we analyzed the potential of the neutrophil-related serum biomarkers NE, NGAL, and LAC in predicting treatment outcomes and disease evolution, in asymptomatic CD patients treated with IFX, over 2 years, in a routine practice setting. Our results suggested that FC (in line with previous reports [19, 20]), but not the serum panel, can predict disease outcomes and

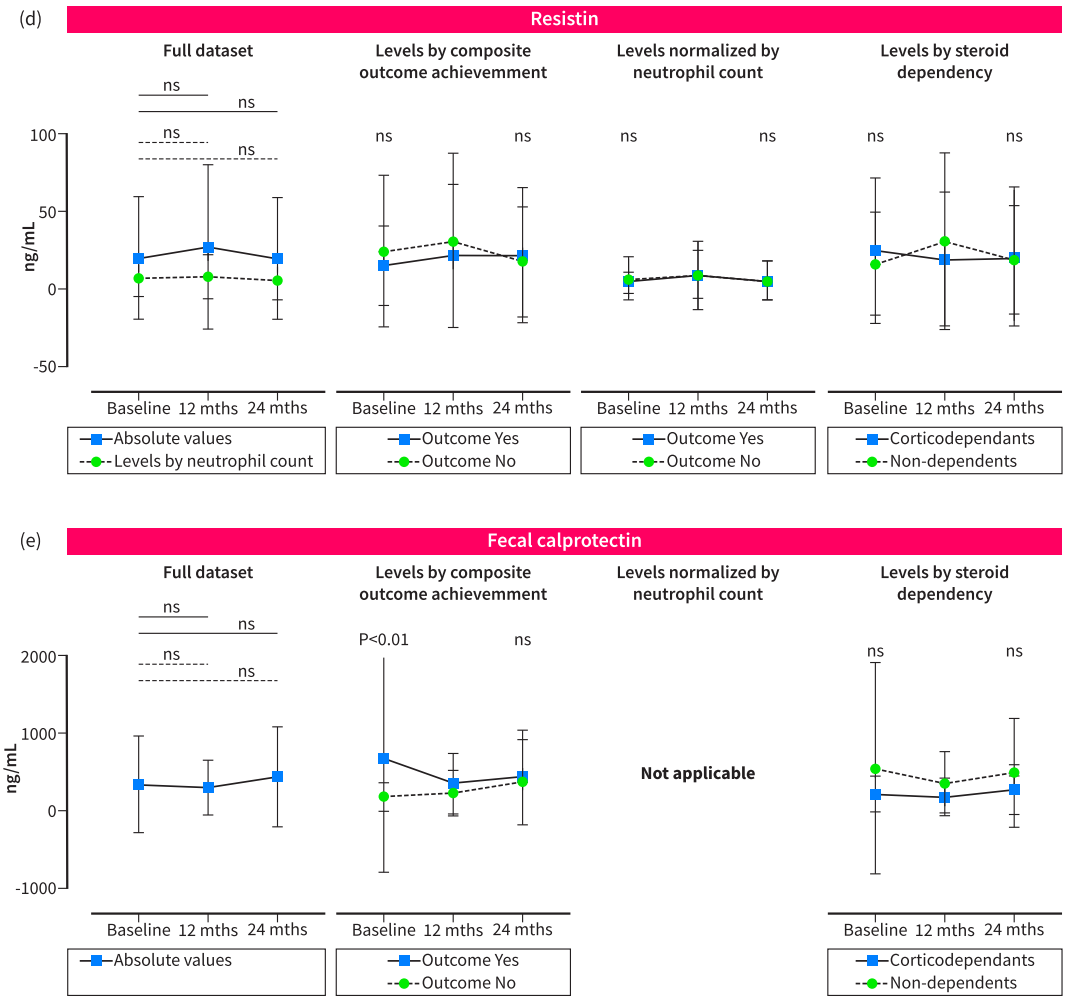


**FIGURE 2** | (Continued)

evolution. Remarkably, we observed that NE, NGAL, and LAC serum concentrations significantly decreased with IFX treatment over 2 years of maintenance treatment, while neutrophil concentrations remained stable. This suggests that IFX might stabilize circulating neutrophils and decrease their degranulation. The direct interplay between IFX and neutrophils has rarely been explored on a mechanistic basis, and only a few prospective studies have looked at its impact on serum neutrophil biomarkers. Recently, Zhang et al. reported for the first time that IFX inhibited the production of calprotectin, reactive oxygen species, and myeloperoxidase in neutrophils isolated from the peripheral blood and inflamed intestinal mucosa of active CD patients treated for 6 weeks [5]. Earlier reviews of the IFX mechanism of action did not mention neutrophils [3].

To our knowledge, this is the largest and longest-term study to assess NE post-IFX. Two small studies reported NE serum levels post-anti-TNFα induction: one observed significantly increased NE serum levels after 2 weeks ($n$ = 11) [21], and the other showed no changes over 14 weeks ($n$ = 21) [22]. NE did not correlate meaningfully with serum IFX, anti-IFX levels, or FC. Most previous cross-sectional studies reported a correlation between serum NE and CD disease activity [23–25], but to our knowledge, none have studied the association between IFX levels and FC. Serum NE values in CD patients were on the lower end of the spectrum of previous reports, which ranged broadly from 0.24 ng/mL [26] to 703 ng/mL [21], with most around 40–200 ng/mL [23, 24, 27]. Notably, all these studies measured NE through an ELISA method, which is known to measure not only free NE, but also NE–α1-AT complexes, with possible increase of measured absolute NE [27, 28]. Our lower levels could be justified by the higher specificity of magnetic bead assays (MBA) to free NE. This trend has been reported in other settings, with poor concurrence between MBA and ELISA values and MBA yielding lower absolute values of cytokines [29, 30].

In our study, NGAL closely followed NE trends. Again, few reports are available in the literature. A numerical decrease of NGAL levels, at 6 months, was observed in CD anti-TNFα responders ($n$ = 17) [13]. In UC, Stallhofer et al. found that IFX significantly lowered NGAL concentrations ($n$ = 60) [31], whereas Komosinska-Vassev et al. observed that adalimumab, over 1 year, did not change NGAL levels ($n$ = 31) [32]. Interestingly, Scoville et al. found significantly lower values in CD patients than in UC patients, but the difference disappeared when anti-TNFα patients were removed, suggesting a reduction due to anti-TNFα, as herein observed [33]. Serum NGAL also mirrored

NE regarding the absence of meaningful relationships with inflammatory markers and HBI, except for CRP. Past reports are conflicting concerning the correlation of NGAL with CD disease activity (yes [13, 34, 35] or no [31, 32, 36]) or CRP (yes [31, 34] or no [32]); our study supports a positive correlation with CRP.

As far as we know, our study is the first one to report longitudinal serum LAC data on IBD. This is surprising, given the importance of fecal LAC in monitoring UC,[37] and that serum LAC has been proposed to reflect gut inflammatory activity in healthy subjects.[38] Cross-sectional studies found higher levels in active versus inactive CD, and in CD versus age- and gender-matched HC [26, 27].

How may IFX influence release of granule content? Degranulation is triggered by binding to GPCR, Fc receptors, or Mac-1 receptors in the plasma membrane, which activate a series of converging intracellular pathways. In one, the Src-family kinase Hck activates the actin-remodeling Rac2 via Syk-DOCK2-Rac2; in another, Hck activates p38 MAPK, which in turn triggers degranulation in a still not fully understood way specifically in primary (where NE is located) and secondary (NGAL and LAC) granules [39]. A third pathway starts by activation of toll-like receptor-9 by microbial nucleic acid PAMPs (pathogen-associated molecular patterns) and includes NF-κB-mediated transcription of iNOS, leading to Hck activation [39]. Dysregulated signaling through toll-like receptors has been proposed to play a role in IBD pathophysiology; it is possible, albeit speculative at this point, that TLR9-related degranulation pathway acquire a specific importance in the IBD context. Both p38 MAPK and NF-κB are upregulated by TNFα (via binding to TNFR1) [3, 5]. Furthermore, degranulation has been shown to be greatly increased in neutrophils previously exposed to TNF; without this priming, the efficacy of other stimuli was reduced [39]. Thus, by blocking TNFα, IFX may indeed reduce neutrophil primary and secondary degranulation.

Why, then, were serum NE, NGAL and LAC not useful in predicting treatment outcomes? It might be that serum biomarkers, in contrast with fecal ones, are either too prone to influence from other systemic disorders or too diluted within the circulation to detect inflammation localized in the intestinal mucosa. Indeed, a recent 12-month-long study found serum calprotectin not useful in predicting relapse in IBD patients in remission treated with biologics [40], despite the wealth of data supporting FC for that purpose [20, 41]. It is also possible that using a composite outcome masked the relationship with a specific endpoint, which coupled with the sample size might have rendered the study unable to find differences.

In our study, resistin was weakly associated with patient-reported HBI and CRP, was not associated with FC, and remained stable over the 2 years of maintenance treatment. A recent meta-analysis found generally higher plasma resistin levels in CD versus HC and in active versus remission, as well as a significant reduction of concentrations post-IFX, despite high heterogeneity [42]. The stability observed during the maintenance period might reflect a relatively mild, asymptomatic population with limited margin for improvement, or that IFX's effect on resistin occurs earlier in the treatment course: patients had an average of 4.1 years on IFX before entering our study, whereas all 3 studies included in the meta-analyses were post-induction and up to 12 months [42].

FC, which acted as a positive control, was significantly higher at the beginning of maintenance follow-up in patients who achieved the disease progression composite outcome. This finding is aligned with the body of evidence that recognizes FC as a prime (yet cumbersome) biomarker to monitor disease progression [6, 19] and IFX treatment outcomes [20, 41] in CD. Moreover, lower FC levels during maintenance regimen were associated with endoscopic response and long-term remission [20, 41]. In this context, our results regarding FC add robustness to the study and credibility to the biomarker serum results.

The main strengths of the study include the real-world setting, long-term duration, centralized lab analyses and a larger population than previous reports longitudinal reports. At the same time, several limitations are acknowledged. First of all, the lack of endoscopic and histological activity endpoints in the composite outcome might have impaired some discriminative ability; for example, the decreasing neutrophil biomarkers could be in part reflecting an incomplete endoscopic response at the beginning of the observation that improved with continuing IFX therapy. Indeed, persistent subclinical inflammation is common in CD [14], and discrepancies between clinical PROs and endoscopic and histological activities are well documented [43], including for PRO-2 [44]. However, an advantage of the composite outcome is that the reappearance of symptoms according to PRO-2 is only one of several items included. Secondly, as an exploratory analysis without formal sample size calculation, it could happen that including more than 100 subjects would be required to find significant results, by reducing data variability. Thirdly, samples endured different storage times; however, results do not suggest an impact of time, and lab assays were standardized. Fourthly, all populations were sourced from the real-world setting and, as such, could differ in non-explored confounding factors. Finally, these results pertain only to asymptomatic IFX-treated CD patients in the maintenance phase, and thus might not be generalizable to patients with UC or other treatment schemes.

Taken together, our real-world data findings do not support the use of NE, NGAL, LAC, or resistin serum concentrations as predictors of treatment outcomes or disease evolution in patients with CD treated with IFX. Instead, our results reinforce the use of FC for that purpose. The age of personalized medicine is upon us, and biomarkers are pivotal in tailoring therapies to each patient. However, to rely on them as treatment guiderails requires extensive and painstaking validation processes. Therefore, ruling out biomarkers is as important as identifying potential new ones and avoiding further efforts devoted to misdirection. Nevertheless, further studies using histological and endoscopic endpoints may provide additional insights. On the other hand, the observed sustained concentration decrease over time suggests a potential additional IFX mode of action via neutrophil degranulation decrease. However, despite biological plausibility based on the known signaling pathways involved in degranulation and TNFR1 activation, this linkage remains speculative at present, and mechanistic studies will be required to better understand this

interplay before definite conclusions can be drawn. Our group is planning assays to address these questions and hope to contribute further to this exciting research field.


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### Conflicts of Interest

Author Fernando Magro served as speaker and received honoraria from Merck Sharp & Dohme, Abbvie, Vifor, Falk, Laboratórios Vitória, Ferring, Hospira and Biogen. All other authors declare no relevant competing interests.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.