Original research

External validation of serum biomarkers predicting short-term and mid/long-term relapse in patients with Crohn's disease stopping infliximab

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► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/gutjnl-2024-332648).

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Received 12 April 2024
Accepted 28 July 2024
Published Online First
12 August 2024

To cite: Pierre N, Huynh-Thu VA, Baiwir D, *et al*. *Gut* 2024;**73**:1965–1973.

ABSTRACT

Objective In patients with Crohn's disease (CD) on combination therapy (infliximab and immunosuppressant) and stopping infliximab (cohort from the study of infliximab diSconTinuation in CrOhn's disease patients in stable Remission on combined therapy with Immunosuppressors (STORI)), the risk of short-term (≤6 months) and mid/long-term relapse (>6 months) was associated with distinct blood protein profiles. Our aim was to test the external validity of this finding in the SPARE cohort (A proSpective Randomized Controlled Trial comParing infliximAb-antimetabolites Combination Therapy to Anti-metabolites monotheRapy and Infliximab monothErapy in Crohn's Disease Patients in Sustained Steroid-free Remission on Combination Therapy).

Design In SPARE, patients with CD in sustained steroid-free clinical remission and on combination therapy were randomly allocated to three arms: continuing combination therapy, stopping infliximab or stopping immunosuppressant. In the baseline serum of the STORI and SPARE (arm stopping infliximab) cohorts, we studied 202 immune-related proteins. The proteins associated with time to relapse (univariable Cox model) were compared between STORI and SPARE. The discriminative ability of biomarkers (individually and combined in pairs) was evaluated by the c-statistic (concordance analysis) which was compared with C-reactive protein (CRP), faecal calprotectin and a previously validated model (CEASE).

Results In STORI and SPARE, distinct blood protein profiles were associated with the risk of short-term (eg, high level: CRP, haptoglobin, interleukin-6, C-type lectin domain family 4 member C) and mid/long-term relapse (eg, low level: Fms-related tyrosine kinase 3 ligand, kallistatin, fibroblast growth factor 2). At external validation, the top 10 biomarker pairs showed a higher c-statistic than the CEASE model, CRP and faecal calprotectin in predicting short-term (0.76–0.80 vs 0.74 vs 0.71 vs 0.69, respectively) and mid/long-term relapse (0.66–0.68 vs 0.61 vs 0.52 vs 0.59, respectively).

Conclusion In patients with CD stopping infliximab, we confirm that the risk of short-term and mid/long-term relapse is associated with distinct blood protein profiles showing the potential to guide infliximab withdrawal.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ In patients with Crohn's disease in infliximab-induced remission, infliximab withdrawal may be contemplated for different reasons (safety concerns, healthcare costs and patient preference) but this decision remains difficult since relapse is largely unpredictable. Previously, we reported that in patients with Crohn's disease stopping infliximab (STORI cohort), the risk of short-term (≤6 months) and mid/long-term (>6 months) relapse was associated with distinct blood protein profiles. The validation of this result in an independent cohort is expected.

WHAT THIS STUDY ADDS

⇒ Validation in an independent cohort (SPARE) of distinct serum biomarkers predicting short-term and mid/long-term relapse in patients with Crohn's disease stopping infliximab.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The validated biomarkers might pave the way for detecting the early phase of Crohn's disease reactivation and, by doing so, they could support or discourage the decision of infliximab withdrawal.

Trial registration number NCT00571337 and NCT02177071.

INTRODUCTION

In Crohn's disease (CD), antitumour necrosis factor-α (TNF-α) treatment can induce long-term sustained steroid-free remission. Once remission is achieved, anti-TNF-α withdrawal may be contemplated for different reasons: safety concerns, healthcare costs and patient preference.[1–3] A meta-analysis demonstrated that, in a homogeneous manner across studies, ~50% of patients with CD in remission and stopping anti-TNF-α do not relapse over

a 2-year period.[4] Thus, a non-negligible part of patients with CD could benefit from a drug holiday. However, relapse remains largely unpredictable, thus making anti-TNF-α withdrawal a difficult decision.[5] In this context, biomarkers predicting relapse are expected.[5]

Previously, we reported that, in patients with CD stopping infliximab (STORI cohort),[6] the risk of short-term (≤6 months) and mid/long-term relapse (>6 months) was associated with distinct blood protein profiles.[7 8] This result needs to be validated in an independent cohort. To this end, the SPARE cohort appears as an ideal and expected validation data set.[9 10] The SPARE study is an open-label, randomised controlled trial where patients with CD in sustained steroid-free clinical remission, receiving combination therapy (infliximab and immunosuppressant), were allocated to three arms: continuing combination therapy, stopping infliximab or stopping immunosuppressant.[11] Thus, in the SPARE cohort, the arm stopping infliximab is equivalent to the STORI trial.

By using the SPARE cohort to validate the biomarkers highlighted in the STORI cohort, the present study aims were: (1) to confirm the distinct blood protein profiles associated with the risk of short-term and mid/long-term relapse; (2) to test the external validity of biomarkers predicting relapse and to compare their performance against the reference biomarkers used in clinical routine practice (C-reactive protein (CRP); faecal calprotectin (FC)) and a validated model including clinical parameters for predicting relapse after anti-TNF-α withdrawal (CEASE model).[12]

METHODS

Patients and protocol

The STORI (n=115) and SPARE (n=211) cohorts have been described previously.[6 11] Briefly, the patients with CD in STORI (from 20 centres in Belgium and France) and SPARE (from 64 centres in Europe and Australia) were in clinical remission (CD activity index (CDAI)<150 and corticosteroid-free >6 months) and had been treated with combination therapy (infliximab and immunosuppressant >12 months in STORI and >8 months in SPARE). In the STORI study, infliximab was withdrawn, while in the SPARE study, patients were randomised to three arms: continuing combination therapy, stopping infliximab or stopping immunosuppressant. Proteins were measured in the baseline samples (sera and faeces) of the STORI and SPARE cohorts. In the STORI study, relapse was defined by a CDAI>250 or between 150 and 250 with an increase of ≥70 points from baseline over two consecutive weeks. In the SPARE study, relapse was defined by either: (1) CDAI≥250 or between 150 and 250 with an increase of ≥70 points over two consecutive visits (1-week apart) and an objective sign of inflammation (CRP>5 mg/L or FC>250 µg/g); (2) new or reopened perianal or enterocutaneous fistula; (3) intra-abdominal abscess (size ≥3 cm) or a perianal abscess (size ≥2 cm); (4) intestinal obstruction confirmed by imaging and requiring hospital admission.

Study design

In the baseline samples of the STORI cohort, we had previously measured 163 proteins:[7 8] (1) 69 in the serum using selected reaction monitoring (SRM); (2) 92 in the serum using proximity extension assay (PEA, immune response panel, Olink); (3) CRP (clinical measurement); (4) FC. In the present study, these measurements were carried out in baseline samples from patients of SPARE who were randomised to the arm stopping infliximab. In addition to testing the external validity of our previously published results (STORI cohort), we generated a new data set (PEA cytokine panel, 45 serum proteins) in the STORI cohort and validated it in the SPARE cohort (arm stopping infliximab).

Proteins measured by SRM were selected from our biomarker discovery pipeline performed in the STORI cohort to find predictors of relapse.[8] Those proteins are mainly related to the inflammatory response (eg, acute-phase response, blood coagulation, complement pathway) (online supplemental table 1). The proteins measured by the PEA immune response and cytokines panels are notably involved in inflammation, adaptive immune response, cytokine-mediated signalling pathways, lymphocyte activation and defence response to the virus (online supplemental table 1).

Five proteins were measured two times (replicates of measurement), as they were included in the two PEA panels (CCL11, CXCL12, IL-6, IL-10) or measured by SRM and in routine clinical practice (CRP). The replicates of measurement were correlated (Spearman's) to evaluate their reproducibility (online supplemental tables 2 and 3). CXCL12 was excluded from analysis due to the low correlation between replicates of measurement (0.52 in STORI and 0.62 in SPARE, online supplemental tables 2 and 3). Thus, the comparison between the STORI and SPARE (arm stopping infliximab) cohorts involved 202 immune-related proteins (online supplemental table 1).

To investigate biomarkers of relapse in a broader context than infliximab withdrawal, we also measured the SRM markers, together with CRP and FC in baseline samples of patients randomised to the other arms of the SPARE study (n=130). Due to sample availability, the proteomic experiments were performed on subsets of the STORI and SPARE cohorts (figure 1).

Selected reaction monitoring

The SRM method was developed in-house as described in online supplemental methods and online supplemental table 4. In SPARE (all arms), the CRP measured in clinical routine (hsCRP) and in SRM showed an excellent correlation (r=0.99) (online supplemental figure 1). A similar result (r=0.96) was obtained in STORI,[8] thus supporting the quality of our technological development. The analytical performance of our SRM method is further shown by our quality controls: intraday precision, interday precision, stability of the instrumental set-up over time, guarantees on target identity and quantotypic properties of the selected peptides (online supplemental methods).

Proximity extension assay

The methods related to the PEA technology have been fully described elsewhere.[13] The analytical performances of each PEA assay are shown on Olink's website (https://www.olink.com/): intra-assay precision, inter-assay precision, limit of detection, lower limit of quantification, upper limit of quantification, hook effect and dynamic range.

Measurement of faecal calprotectin and CRP

FC was measured by ELISA (PhiCal test, Calpro AS, Norway) in STORI and by turbidimetric immunoassay (Bühlmann fCAL turbo test, Switzerland) in SPARE. CRP was measured by SRM and by high-sensitivity turbidimetric immunoassay (hsCRP) in STORI and SPARE.

Statistical analysis

Results are reported according to the Transparent Reporting of a multivariable prediction model for Individual

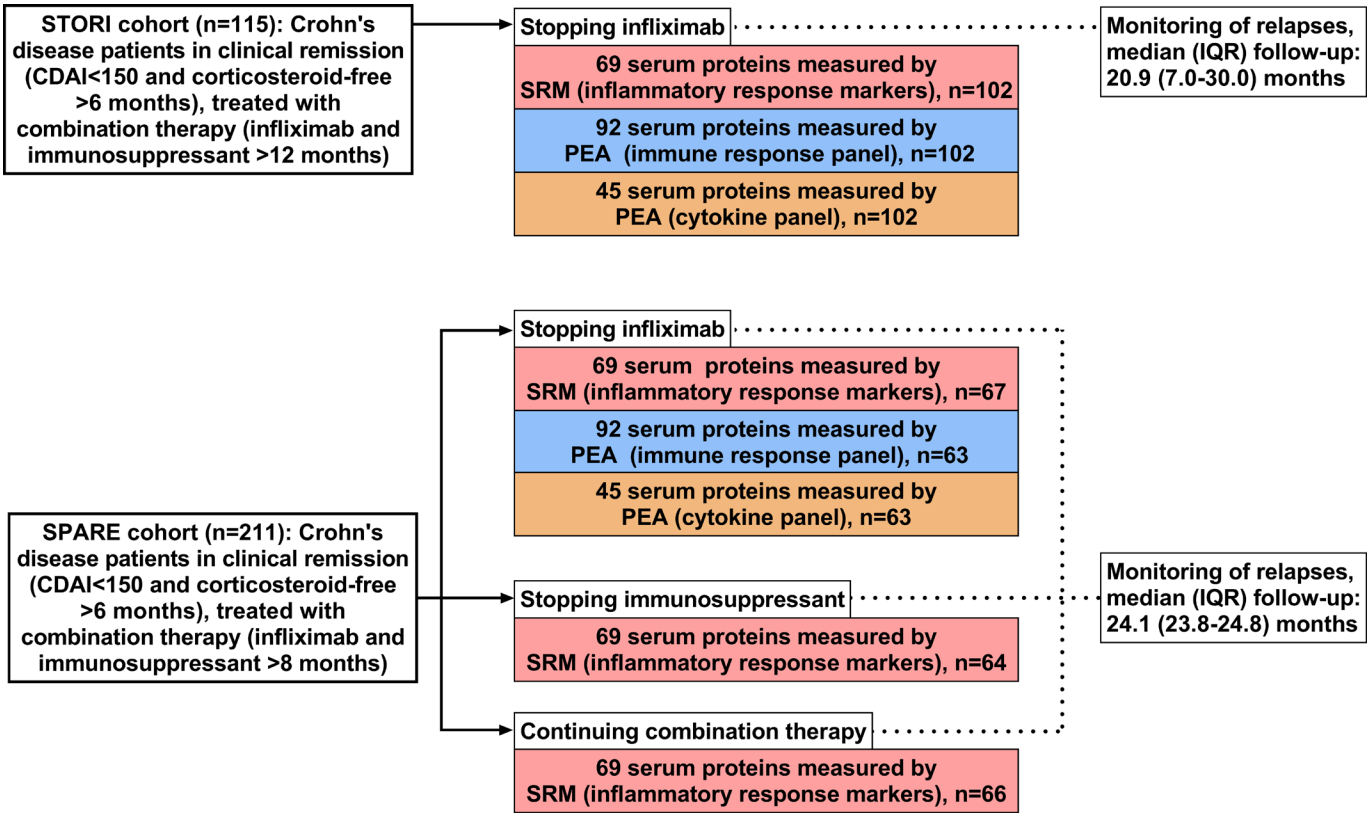

Figure 1 Experimental design. CDAI, Crohn's disease activity index; PEA, proximity extension assay; SRM, selected reaction monitoring.

Prognosis or Diagnosis statements.[14] For the follow-up data, days were converted to months using the following formula: 1 day=0.0328767 months. As previously described,[7 8] proteins associated with the risk of short-term (≤6 months) and mid/long-term relapse (>6 months) were investigated by using time to relapse and time of follow-up to generate two data sets from the original one: (1) short-term relapse data set in which mid/long-term relapsers were classified as non-relapsers and the follow-up of non-relapsers was censored at 6 months; (2) mid/long-term relapse data set in which short-term relapsers and non-relapsers with a follow-up ≤6 months were excluded.

To characterise the blood protein profiles associated with the different times to relapse, the univariable Cox model was applied as previously described.[7 8] The reproduction of this analysis is intended to generate results comparable to our previous studies.[7 8] Briefly, the univariable Cox model was used to associate the time to relapse with the dichotomised level of each protein. The cut-offs for each protein level were optimised separately in STORI and in SPARE using the maximum of Youden's Index. The Cox model generated the hazard ratio (HR) and its associated statistics. The Cox proportional hazard assumption was checked graphically (crossing of the Kaplan-Meier curves) and using the Schoenfeld residuals test. The variables violating this assumption were excluded. The biological convergences between STORI and SPARE were investigated by selecting proteins respecting the following criteria: (1) HR with p value<0.1 in the short-term relapse data set or mid/long-term relapse data set of STORI and SPARE; (2) HR having the same direction (<1 or >1) in the short-term relapse data set or mid/long-term relapse data set of STORI and SPARE.

The external validation of biomarkers was restricted to proteins showing biological convergences between the two cohorts (see above) and this was performed with the following procedure. The level of each protein was first standardised to zero mean and unit variance. By using the continuous and standardised levels of proteins (individually and systematically combined in pairs), the coefficients of the Cox models were fitted to the STORI cohort (development data set) and they were then applied to the SPARE cohort (validation data set). Similarly, the Cox models were fitted to the SPARE cohort (development data set) and applied to the STORI cohort (validation data set). The discriminative ability of biomarkers was evaluated using the mean of Harrel's c-statistic[15] obtained in the validation data sets (STORI and SPARE). As previously defined:[15] 'Discrimination measures a predictor's ability to separate patients with different responses'. The c-statistic provides values between 0 and 1, where 0.5 and 1 correspond to random and perfect discrimination, respectively. The 95% CI of the c-statistic was obtained with a stratified bootstrap procedure (n=500) performed on the development data sets. The discriminative ability of biomarkers was compared with the one obtained with CRP, FC and the CEASE model including nine clinical variables:[12] age (per 10 years); smoking (yes vs no); age at diagnosis (per 5 years); upper gastrointestinal tract involvement (L4 vs non-L4); immunosuppressant (yes vs no); type of anti-TNF used (infliximab vs adalimumab); second-line anti-TNF (yes vs no); CRP (per doubling) and FC (per doubling).

The correlations were determined by Spearman's rank test. All statistical tests were two-sided. The statistical analyses were conducted using Python V.3.9, R V.3.3.1 and GraphPad Prism V.9.4.1. The Cox survival analysis was performed using the Python library lifelines 0.27.[16] The heat maps and volcano plots were generated using the ggplot2 R package.[17]

Table 1 Patients' characteristics and follow-up

	STORI (n=115)	SPARE, infliximab withdrawal (n=71)	SPARE, immunosuppressant withdrawal (n=69)	SPARE, combination therapy (n=71)
Age (years), median (IQR)	32 (26–39)	32 (25–42.5)	31 (26–44)	37 (27–45.5)
Gender				
Female	66 (57%)	28 (39%)	31 (45%)	33 (46%)
Male	49 (43%)	43 (61%)	38 (55%)	38 (54%)
Disease duration (years), median (IQR)	7.8 (4.5–11.8)	6.7 (3.3–10.7)	6.8 (2.9–12.6)	6.4 (3.2–12.7)
Active smoker	45 (39%)	14 (20%)	17 (25%), n=68	17 (24%)
Disease location				
Ileal (L1)	14 (12%)	10 (14%)	13 (19%)	15 (21%)
Colonic (L2)	36 (31%)	20 (28%)	21 (30%)	24 (34%)
Ileocolonic (L3)	64 (56%)	41 (58%)	35 (51%)	32 (45%)
Upper gastrointestinal tract (L4)	9 (8%)	8 (11%)	5 (7%)	10 (14%)
Previous intestinal resection	25 (22%)	13 (18%)	17 (25%)	13 (18%)
Steroid treatment between 12 and 6 months before screening	8 (7%)	1 (1%)	3 (4%)	6 (8%), n=69
CDAI, median (IQR)	37 (20–61)	38 (18–57.5)	42 (25–83)	53 (25–77.5)
Treatment history				
Methotrexate	19 (17%)	2 (3%)	6 (9%)	5 (7%)
Azathioprine/mercaptopurine	96 (83%)	69 (97%)	63 (91%)	66 (93%)
Duration of infliximab treatment (years), median (IQR)	2.2 (1.5–3.1)	2.5 (1.4–4.5)	2.6 (1.6–4.0)	2.3 (1.5–3.6)
Endoscopy				
CDEIS, median (IQR)	0.7 (0.0–2.9)	0 (0–0)	0 (0–0), n=68	0 (0–0)
CDEIS=0	40 (35%)	63 (89%)	57 (83%)	58 (82%)
Remaining ulcers	39 (34%)	8 (11%)	6 (9%)	8 (11%)
Biological variables				
Haemoglobin level, g/L, median (IQR)	135 (128–144)	142 (137–151)	140 (133–149)	140 (132–145)
Haematocrit, %, median (IQR)	40 (37–43)	41 (40–44)	41 (39–44)	41 (39–43.5)
Leucocyte count, 10^9/L, median (IQR)	6.1 (5.0–7.5)	5.3 (4.5–7.0)	6.3 (5.3–7.6)	5.5 (4.9–6.8)
Platelet count, 10^9/L, median (IQR)	272 (233–313)	256 (216–299), n=70	254 (215–302)	244 (217–307)
hsCRP level, mg/L, median (IQR)	2.0 (0.9–4.8), n=109	1.2 (0.6–2.3), n=69	1.2 (0.5–2.6), n=66	1.2 (0.5–2.8), n=69
Faecal calprotectin, µg/g, median (IQR)	51 (30–318), n=85	95 (22–289), n=47	61 (22–195), n=53	77 (25–315), n=44
Infliximab trough level, mg/L, median (IQR)	3.7 (1.7–8.0)	4.1 (2.5–6.3), n=69	4.1 (2.4–5.7), n=66	3.6 (2.6–5.9), n=69
Follow-up				
Number of relapsers	52 (45%)	25 (35%)	6 (9%)	8 (11%)
Number of short-term relapsers (<6 months)	21 (40%)	10 (40%)	2 (33%)	2 (25%)
Number of mid/long-term relapsers (>6 months)	31 (60%)	15 (60%)	4 (67%)	6 (75%)
Number of non-relapsers	63 (55%)	46 (65%)	63 (91%)	63 (89%)
Time to relapse of short-term relapsers (months), median (IQR)	3.8 (2.8–4.4)	3.5 (3.3–3.9)	4.6 (4.2–5.1)	3.7 (2.8–4.6)
Time to relapse of mid/long-term relapsers (months), median (IQR)	8.2 (6.8–12.1)	10.3 (7.9–12.6)	19.1 (18.2–20.4)	19.0 (16.9–20.7)
Time of follow-up of non-relapsers (months), median (IQR)	20.9 (7.0–30.0)	24.1 (23.8–24.8)	23.9 (21.6–24.3)	23.9 (17.6–24.2)

CDAI, Crohn's disease activity index; CDEIS, Crohn's disease endoscopic index of severity.

RESULTS

Study population and follow-up in STORI and SPARE (arm stopping infliximab)

The clinical characteristics and the follow-up data of the STORI and SPARE cohorts are presented in table 1. In STORI and SPARE (arm stopping infliximab), the relapse rate was 45% (52/115) and 35% (25/71) over a median follow-up of 20.9 and 24.1 months, respectively. As shown by the IQR, the follow-up showed higher variability in STORI than SPARE (arm stopping infliximab) (7.0–30.0 vs 23.8–24.8, respectively). This difference is due to the fact that the SPARE protocol included a follow-up duration of 2 years while, in STORI, the end of follow-up was the date of the closure of the study. In STORI and SPARE (arm stopping infliximab), the majority of relapses occurred more than 6 months after stopping infliximab (STORI: 60% (31/52); SPARE: 60% (15/25)). The patients from STORI and SPARE (arm stopping infliximab) had close median times of short-term relapse (3.8 vs 3.5 months, respectively) and mid/long-term relapse (8.2 vs 10.3 months, respectively).

Proteins associated with the risk of short-term and mid/long-term relapse in the STORI and SPARE cohorts

The results from the STORI and SPARE (arm stopping infliximab) cohorts converged to show distinct blood protein profiles associated with the risk of short-term and mid/long-term relapse (figures 2–4). Similar results were obtained when STORI was

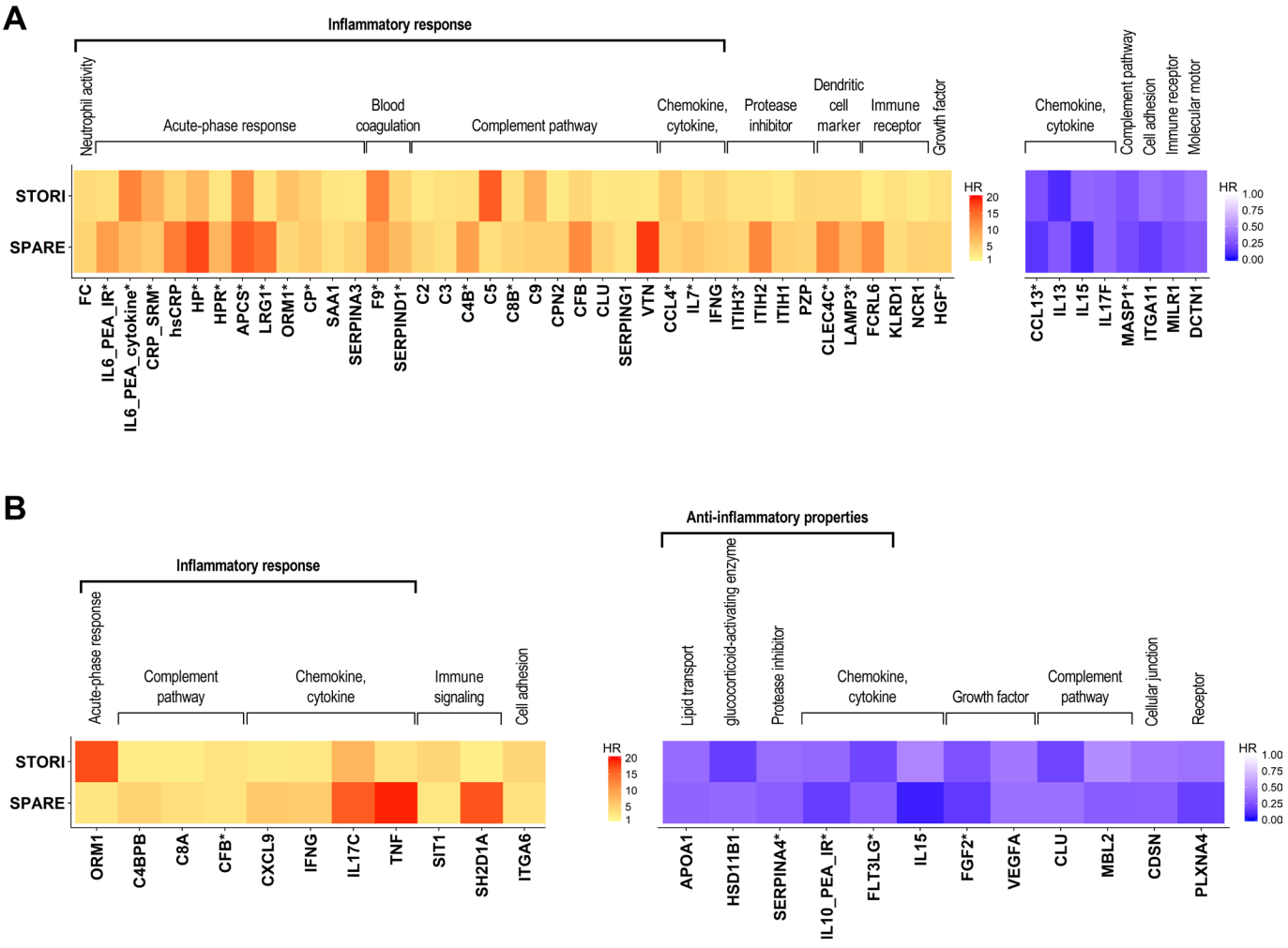

Figure 2 Proteins associated with the risk of short-term and mid/long-term relapse in the STORI and SPARE (arm stopping infliximab) cohorts. (A) Risk of short-term relapse (≤6 months). (B) Risk of mid/long-term relapse (>6 months). P value<0.1 in STORI and SPARE; *p value<0.05 in STORI and SPARE. CRP_SRM: CRP measured by SRM; hsCRP: CRP measured by high-sensitivity turbidimetric immunoassay; IL-6_PEA_IR: IL-6 measured by the PEA immune response panel; IL-6_PEA_cytokine: IL-6 measured by the PEA cytokine panel; IL-10_PEA_IR: IL-10 measured by the PEA immune response panel; IL-10_PEA_cytokine: IL-10 measured by the PEA cytokine panel. CRP, C-reactive protein; IL, interleukin; PEA, proximity extension assay; SRM, selected reaction monitoring.

compared with all arms of SPARE (analysis restricted to SRM markers) (online supplemental figures 2–4). Five proteins (CFB, CLU, IFNG, IL-15, ORM1) were associated with both the risk of short-term and mid/long-term relapse in STORI and SPARE (arm stopping infliximab) (figure 2). Of note, CLU presented an opposite association (HR>1 vs HR<1) for the risk of short-term and mid/long-term relapse (figure 2). The convergent results between STORI and SPARE (arm stopping infliximab) were more numerous for proteins associated with the risk of short-term relapse than for those associated with the risk of mid/long-term relapse (45 vs 23) (figure 2). A similar observation was found when STORI was compared with all arms of SPARE (analysis restricted to SRM markers) (online supplemental figures 2–4).

In STORI and SPARE (arm stopping infliximab), a high level of inflammatory response proteins was strongly associated with the risk of short-term relapse (27 markers, among which 14 had HR with p value<0.05 in the two cohorts) and weakly associated with the risk of mid/long-term relapse (8 markers, among which 1 (CFB) had HR with p value<0.05 in the two cohorts) (figure 2). The risk of mid/long-term relapse was specifically associated with a low level of proteins having anti-inflammatory properties (APOA1, HSD11B1, SERPINA4, IL-10, FLT3LG) (figure 2). Of note, the result for IL10 was obtained with the PEA immune response panel. When IL-10 was measured with the PEA cytokine panel, this led to the same conclusion in STORI but not in SPARE (arm stopping infliximab) (figure 4).

External validation of biomarkers

The selected serum proteins (figure 2) along with the CEASE model, CRP and FC were subjected to external validation (see Methods). All these results are presented in online supplemental tables 5–10. The top 10 pairs of biomarkers showed a higher c-statistic (discriminative ability) than the CEASE model, CRP and FC to predict short-term (0.76–0.80 vs 0.74 vs 0.71 vs 0.69, respectively) and mid/long-term relapse (0.66–0.68 vs 0.61 vs 0.52 vs 0.59, respectively) (table 2). The combination of SIT1 and HP showed a higher c-statistic than the CEASE model, CRP and FC in predicting relapse during the whole follow-up period (0.67 vs 0.66 vs 0.62 vs 0.64, respectively) (table 2). Of note, HP and FLT3LG were over-represented in the best pairs of biomarkers (table 2) and, when taken individually, these proteins showed the highest c-statistic for

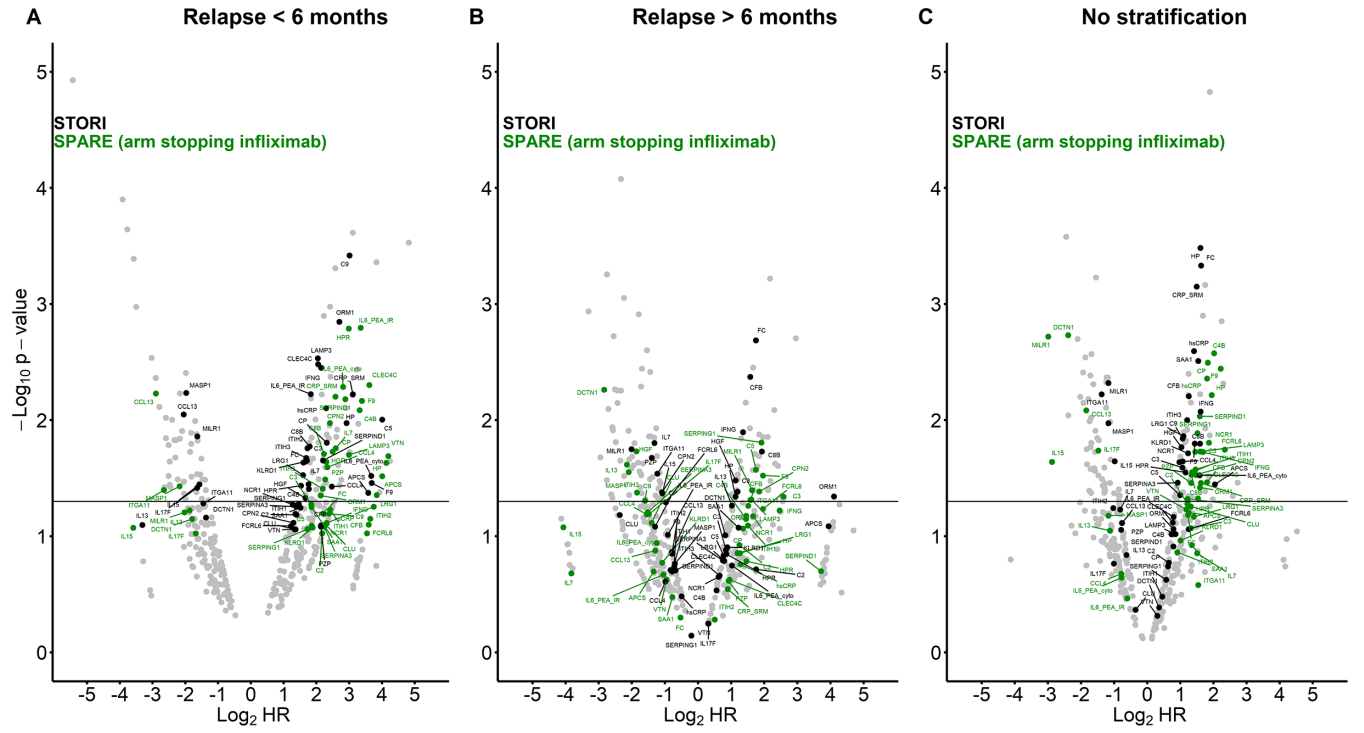

Figure 3 Proteins associated with the risk of short-term relapse in STORI and SPARE (arm stopping infliximab). In STORI and SPARE (arm stopping infliximab), the association between the level of each protein and the time to relapse was depicted in volcano plots ((A) short-term relapse data set; (B) mid/long-term relapse data set; (C) non-stratified data set) with the x-axis representing the magnitude of the risk (log_2 HR) and the y-axis its degree of significance ($-log_{10}$ p value). The horizontal lines correspond to p value=0.05. CRP_SRM: CRP measured by SRM; hsCRP: CRP measured by high-sensitivity turbidimetric immunoassay; IL-6_PEA_IR: IL-6 measured by the PEA immune response panel; IL-6_PEA_cytokine: IL-6 measured by the PEA cytokine panel. CRP, C-reactive protein; IL, interleukin; PEA, proximity extension assay; SRM, selected reaction monitoring.

predicting short-term (0.74) and mid/long-term relapse (0.66), respectively (online supplemental tables 8 and 9, respectively). Finally, the c-statistic of the top 10 pairs of biomarkers, CEASE model, CRP and FC were higher for predicting short-term than mid/long-term relapse (table 2).

DISCUSSION

By studying 202 serum proteins and FC in two independent cohorts (STORI and SPARE), we confirmed that, in patients with CD stopping infliximab, the risk of short-term and mid/long-term relapse is associated with distinct biological profiles. We also highlighted the potential of novel serum biomarkers over clinical parameters to guide the decision of anti-TNF-α withdrawal.

Overall, our study showed that predicting short-term and mid/long-term relapse requires targeting proteins involved in different functions. The risk of short-term relapse is mainly predicted by inflammatory proteins such as those used in clinical routine (CRP and FC), while predicting mid/long-term relapse would require targeting proteins involved in different functions (eg, anti-inflammatory and wound healing). The prediction of mid/long-term relapse has a practical interest since, after stopping infliximab, the majority of relapsers showed a time to relapse superior to 6 months (60% in STORI and SPARE). Finally, from a conceptual point of view, predicting short-term and mid/long-term relapse could make clinical decision-making more granular by guiding different therapeutic strategies.[18]

Inflammation-related proteins were strongly associated with the risk of short-term relapse and weakly associated with the risk of mid/long-term relapse. This observation highlights the continuous progression of inflammation before relapse and, by doing so, it supports that our methodological approach (stratification based on survival data) can capture dynamic information from cross-sectional samples (baseline).

The converging results between the two cohorts were more numerous and marked for proteins associated with the risk of short-term relapse than for those associated with the risk of mid/long-term relapse. This observation appears logical and intuitive when considering the disease dynamics. It is very likely that biological dysfunctions become more prominent and homogeneous as relapse approaches. Indeed, whatever its trigger, the relapse process ends with an inflammatory flare, making the biological profile associated with the risk of short-term relapse robust. On the other hand, biological dysfunctions preceding the inflammatory flare could be more discreet and heterogeneous, making the biological profile associated with the risk of mid/long-term relapse more difficult to capture. Indeed, this state being closer to relapse triggers, it could strongly depend on individual interactions between genetics and environmental factors.

The biological condition of mid/long-term relapsers is of interest, as it represents an early phase of CD reactivation, that is, the one preceding the inflammatory flare (short-term relapsers). We reported associations between the risk of mid/long-term relapse and the low serum level of FLT3LG, FGF2, IL-10 and SERPINA4. FLT3LG is a cytokine that promotes the proliferation of haematopoietic stem cells, along with the expansion and function of conventional dendritic cells and plasmacytoid dendritic cells (pDC).[19] Of note, we observed that CLEC4C, a specific marker of pDC,[20] was associated with the risk of short-term relapse in STORI and SPARE. In a mice model of ileitis, administration of FLT3LG reduced the disease severity, and led to an expansion of the pro-regulatory DC

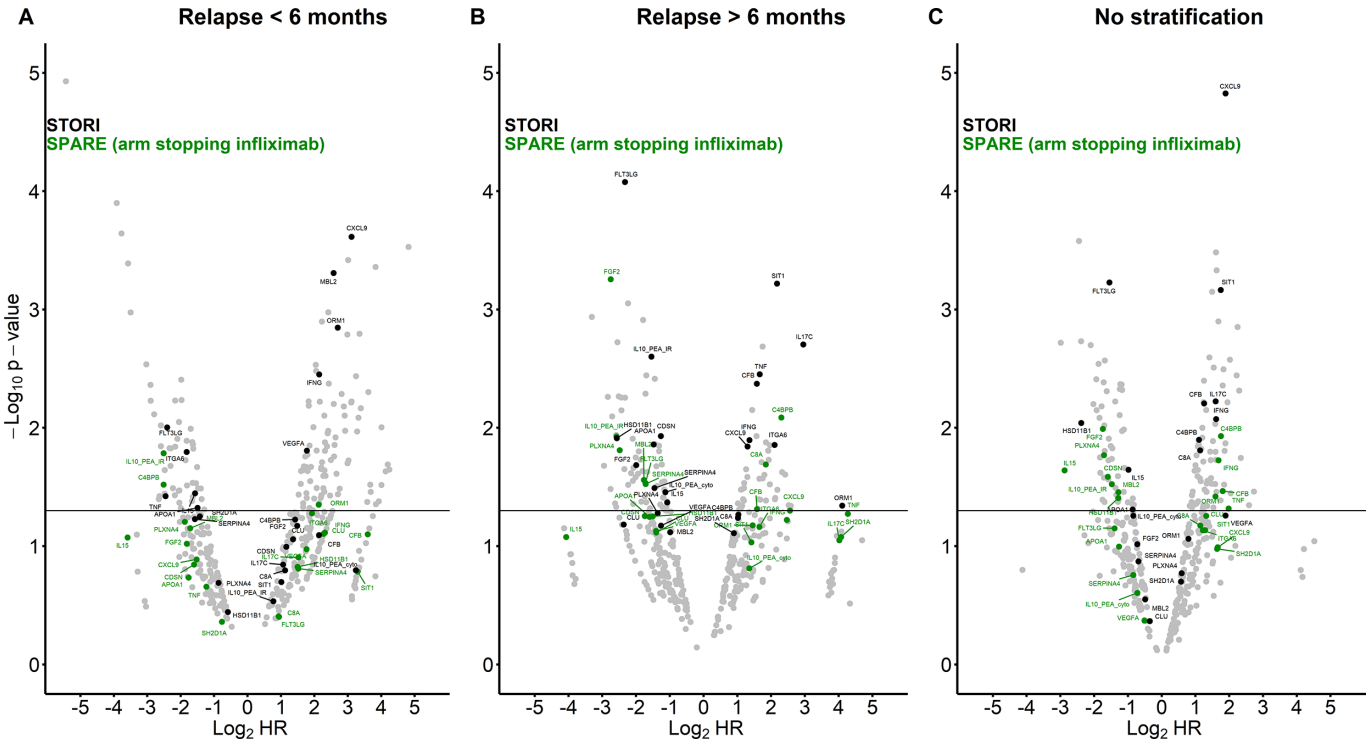

Figure 4 Proteins associated with the risk of mid/long-term relapse in STORI and SPARE (arm stopping infliximab). In STORI and SPARE (arm stopping infliximab), the association between the level of each protein and the time to relapse was depicted in volcano plots ((A) short-term relapse data set; (B) mid/long-term relapse data set; (C) non-stratified data set) with the x-axis representing the magnitude of the risk (log_2 HR) and the y-axis its degree of significance ($-log_{10}$ p value). The horizontal lines correspond to p value=0.05. IL-10_PEA_IR: IL-10 measured by the PEA immune response panel; IL-10_PEA_cytokine: IL-10 measured by the PEA cytokine panel. CRP, C-reactive protein; IL, interleukin; PEA, proximity extension assay; SRM, selected reaction monitoring.

($CD103^+$ DC) and regulatory T cells ($CD4^+/CD25^+/FoxP3^+$) in the lamina propria.[21] Thus, FLT3LG seems to exert potent anti-inflammatory effects in the gut.[21] FGF2 is a growth factor that promotes wound healing of the intestinal epithelium via its action on stem cell survival, epithelial cell proliferation and restitution.[22] Remarkably, FGF2 administration (recombinant) and FGF2 genetic ablation alleviated and worsened dextran sulfate sodium (DSS)-induced colitis, respectively.[22 23] Mechanistically, it has been shown that transforming growth factor-β1 stimulates the production of FGF2 by lamina propria regulatory T cells during DSS-induced colitis.[23] Then, cooperation between the FGF2 and IL-17 signalling promotes epithelium wound healing.[23] IL-10 has potent and broad anti-inflammatory actions which are indispensable to maintain intestinal immune tolerance.[24] SERPINA4 is mainly produced by the liver, it has anti-inflammatory, anti-oxidative and anti-apoptotic effects.[25] Although the role of SERPINA4 in mucosal homeostasis is unknown, its low serum level has been associated with the preclinical phase of CD (complication at diagnosis).[26] Considering the available evidence, we hypothesise that a defect in wound healing mechanisms and a reduction of anti-inflammatory capacities precede the inflammatory flare.

Our markers converged with those highlighted in the preclinical phase of CD (PREDICT and GEM projects). 16 proteins predicting CD (up to 5 years before diagnosis)[27 28] and/or complication at diagnosis (B2, B3 phenotypes or surgery)[26] were also associated with the risk of relapse in STORI and SPARE (CRP, APCS, SAA1, SERPINA3, SERPIND1, SERPINA4, C5, C6, C9, CFH, CFB, CFI, HGF, CXCL9, IL-10, IL-7). This observation could suggest that disease reactivation after a sustained remission at least partly follows the same pathways as during the preclinical phase of disease occurrence.

The generalisability of the highlighted biomarkers needs to be further evaluated. This perspective provides an opportunity to perform a 'strong external validation',[29] that is, validation in various clinical settings such as: (1) relapse after withdrawal of other anti-TNF-α therapies than infliximab (eg, adalimumab, certolizumab pegol); (2) relapse after withdrawal of biologics others than anti-TNF-α (anti-α4β7 integrin, anti-IL-12/IL-23 and anti-IL-23); (3) relapse in ulcerative colitis.

Our study has several strengths. Our data sets[30 31] can be verified, reused and our work meets the need for validation studies in biomarker research.[32] Furthermore, we studied well-characterised and comparable prospective cohorts with robust technologies (SRM and PEA).[13 33] The SRM method was developed in-house and we provide evidence of its specificity and precision (mean intraday variation=5%, mean interday variation=15%). PEA is a proven technology with high specificity[13] and precision (PEA immune response panel: mean intra-assay variation=9%, mean inter-assay variation=17%; PEA cytokine panel: mean intra-assay variation=4%, mean inter-assay variation=6%). PEA and mass spectrometry-based proteomics are complementary technologies. In blood, their combination allows to study various biological processes by increasing proteome coverage.[34] Indeed, SRM mainly targets high-abundant proteins (down to ~1 µg/mL)[33] while PEA allows to investigate low-abundant proteins (down to ~1 pg/mL).[34]

Our work has some limitations. The proportion of missing data was relatively high for FC. Second, the lack of replication of the IL-10 finding in the SPARE cohort. This result being

Table 2 Discriminative ability of the top 10 biomarker pairs, faecal calprotectin, C-reactive protein and CEASE model for predicting relapse

	Gene names 1	Gene names 2	Mean c-statistic (CI) validation data sets (STORI and SPARE), n=SPARE-STORI
Short-term relapse (<6 months)	CLEC4C	ITGA11	0.80 (0.76 to 0.81), n=63–102
	FC	HP	0.79 (0.70 to 0.81), n=44–77
	FC	F9	0.78 (0.67 to 0.79), n=44–77
	CLEC4C	SAA1	0.77 (0.53 to 0.79), n=63–101
	IL-6_PEA_IR	HP	0.77 (0.66 to 0.78), n=63–101
	KLRD1	HP	0.77 (0.74 to 0.78), n=63–101
	ITGA11	HP	0.77 (0.74 to 0.78), n=63–101
	CLEC4C	HP	0.77 (0.73 to 0.78), n=63–101
	CLEC4C	CRP_SRM	0.77 (0.66 to 0.78), n=63–101
	IL-6_PEA_ cytokine	HP	0.76 (0.71 to 0.79), n=63–102
	CEASE model	NA	0.74, n=46–83
	hsCRP	NA	0.71, n=69–109
	FC	NA	0.69, n=47–85
Mid/long-term relapse (>6 months)	FLT3LG	TNF	0.68 (0.65 to 0.69), n=53–73
	SIT1	FLT3LG	0.67 (0.60 to 0.68), n=53–73
	FLT3LG	SERPINA4	0.67 (0.57to 0.69), n=53–73
	CXCL9	FLT3LG	0.67 (0.55 to 0.69, n=53–73
	FLT3LG	IFNG	0.67 (0.55 to 0.70), n=53–73
	FLT3LG	IL-17C	0.66 (0.53 to 0.67), n=53–73
	FGF2	FLT3LG	0.66 (0.58 to 0.71), n=53–73
	FLT3LG	C8A	0.66 (0.55 to 0.67), n=53–73
	FGF2	ORM1	0.66 (0.49 to 0.67), n=53–73
	IL-10_PEA_IR	FLT3LG	0.66 (0.61 to 0.68), n=53–73
	CEASE model	NA	0.61, n=40–56
	FC	NA	0.59, n=41–57
	hsCRP	NA	0.52, n=57–76
Relapse in the non-stratified data set	SIT1	HP	0.67 (0.61 to 0.68) n=63–101
	CEASE model	NA	0.66, n=46–83
	HP	APOA1	0.66 (0.62 to 0.68), n=67–102
	FLT3LG	HP	0.66 (0.63 to 0.67), n=63–102
	hsCRP	HP	0.66 (0.59 to 0.67), n=67–98
	CRP_SRM	HP	0.66 (0.58 to 0.67), n=67–102
	MILR1	HP	0.65 (0.55 to 0.67), n=63–101
	HP	SAA1	0.65 (0.58 to 0.66), n=67–102
	HP	C2	0.65 (0.60 to 0.66), n=67–102
	FC	HP	0.65 (0.44 to 0.69), n=44–77
	hsCRP	SIT1	0.65 (0.58 to 0.67), n=63–98
	FC	NA	0.64, n=47–85
	hsCRP	NA	0.62, n=69–109

NA, not applicable.

particularly important, its validity needs to be clarified in independent studies. Third, we did not collect data on ethnicity. Given the centres involved in patient recruitment, it is very likely that our results were mainly collected from European populations. The generalisation of our findings to other populations needs to be investigated.

In summary, we confirm that the risk of short-term and mid/long-term relapse is associated with distinct blood protein profiles in patients with CD stopping infliximab. The risk of short-term relapse is mainly associated with a high level of inflammatory proteins while the risk of mid/long-term relapse is notably associated with a low level of anti-inflammatory proteins. We also externally validated serum biomarkers predicting relapse and showed their potential for clinical translation and guidance in treatment de-escalation.

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Acknowledgements SPARE trial was funded by the European Union's Horizon 2020 research and innovation programme, under the grant agreement number 633168—BIOCYCLE (PHC-13-2014). The members of SPARE Biocycle research group collaborative authorship can be found in the appendix. Sylvie Chevret wrote the statistical plan. GETAID was the main promotor of the SPARE clinical trial and was the promotor of the trial in France. GETAID gave delegation for specific national tasks (including, local submission to ethics committee and national health authorities, national insurances and centres monitoring) to national co-promotors. CHU Liège (Liège, Belgium), The Skane University Hospital (Lund, Sweden), The AMC Medical Research (Amsterdam, Netherlands), The CDE Service GMBH (Kiel, Germany), the University of Edinburgh (Edinburgh, UK) and St-Vincent Hospital (Melbourne, VIC, Australia) were the national co-promotors. The GETAID, the Swedish Organization for the study of IBD (SOIBD) and the Belgian IBD research and development (BIRD) actively participated in the study (trial protocol reviewing and centres selection in France, Sweden and Belgium).

Collaborators The following GETAID centres (investigators) participated in the STORI trial: Amiens (Jean-Louis Dupas), Bordeaux (David Laharie), Caen (Jean-Marie Reimund), Clichy-Beaujon (Yoram Bouhnik), Colombes-L Mourier (Pauline Jouet), Gent (Martine De Vos), Liège (Jacques Belaiche, Edouard Louis), Lille (Jean-Frédéric Colombel, Gwenola Vernier-Massouille), Lyon (Stéphane Nancey), Marseille (Jean Charles Grimaud), Montpellier (Michel Veyrac), Nantes (Arnaud Boureille, Mathurin Flamant), Paris-HEGP (Raymond Jian), Paris-Lariboisière (Philippe Marteau), Paris-St Louis (Marc Lemann, Matthieu Allez), Rouen (Guillaume Savoye), Strasbourg (Bernard Duclos) and Tours (Laurence Picon). Members of the SPARE Biocycle research group collaborative authorship: A Andrews, M Sparrow, R Leong, S Connor, G Radforth-Smith, P De Cruz, F Baert, E Louis, P Bossuyt, M Resche-Rignon, N Ding, S Almer, S Ben-Horin, JF Colombel,B Siegmund, J Preiss, A Stallmach, T Liceni, O Grip, J Halfvarson, D Durai, F Cummings, C Seilinger, M Parkes, J Lindsay, G Lambrecht, P VanHootegem, JF Rahier, M Dewitte, X Hebuterne, E Chanteloup, R Altwegg, S Nancey, G Bouguen, G Pineton de Chambrun, F Pollenot, Y Bouhnik, L Vuitton, M Nachury, D Laharie, S Nancey, M Fumery, G Bouguen, L Picon, C Gilletta, S Viennot, X Roblin, J Satsangi, J Lindsay, M Parkes, P Irving, C Lamb, D Durai, R Pollok, G D'haens, E Hertervig, O Grip, J Halfvarsson.

Contributors EL and J-FC conceived the STORI and SPARE trials. EL, DL, J-FC, PB, LV and JS were involved in the recruitment of patients and their follow-up. NP developed the selected reaction monitoring (SRM) method with the help of M-AM and DB. NP and LT performed the sample preparation for the SRM analysis. DB, GM, MF, LT, GE and EDP managed the instrumental set-up for the SRM analysis. NP, SV and VAH-T verified and curated the data. VAH-T and NP performed the statistical analysis. NP created the figures. NP wrote the manuscript. EL, M-AM, SV, VAH-T, PB, JS and J-FC critically reviewed the manuscript. NP is the guarantor.

Funding This work was supported by the European Union's Horizon 2020 under the grant agreement N° 633168 — BIOCYCLE (PHC-13-2014), the Walloon Region and the Fond Européen de Développement Régional (FEDER) (Grant portfolio 246099-510388) and internal funding from the Liege University Hospital.

Competing interests PB has received research grants from AbbVie, Amgen, Celltrion, Mylan, Pfizer and Takeda; lecture fees from AbbVie, Celltrion, Janssen, Lilly and Takeda; and consulting fees from AbbVie, Arena Pharmaceuticals, Bristol Myers Squibb, Celltrion, Dr Falk Pharma, Galapagos, Janssen, Lilly, Pentax, PSI-CRO, Roche, Takeda and Tetrameros. LV has received fees from AbbVie, Amgen, Biogen, Mylan, Takeda, MSD, Janssen, Pfizer, Ferring and Galapagos. SV has received speaker's fees from AbbVie, Ferring, Janssen and Takeda. J-FC has received grants from AbbVie,

Janssen Pharmaceuticals, Prometheus, Takeda and Bristol Myers Squibb; receiving payment for lectures from AbbVie, and Takeda; receiving consulting fees from AbbVie, Amgen, AnaptysBio, Allergan, Arena Pharmaceuticals, Boehringer Ingelheim, Bristol Myers Squibb, Celgene Corporation, Celltrion, Eli Lilly, Ferring Pharmaceuticals, Galmed Research, Glaxo Smith Kline, Genentech (Roche), Janssen Pharmaceuticals, Kaleido Biosciences, Immunic, Invea, Iterative Scopes, Merck, Landos, Microba Life Science, Novartis, Otsuka Pharmaceutical, Pfizer, Protagonist Therapeutics, Prometheus, Sanofi, Seres, Takeda, Teva, TiGenix, Vifor; and hold stock options in Intestinal Biotech Development. EL has received educational and research grants from Janssen, Pfizer, AbbVie and Takeda, Fresenius-Kabi; speaker fees from AbbVie, Dr Falk Pharma, Ferring, Janssen, Pfizer, Celgene, Bristol Myers Squibb (BMS), Galapagos and Takeda; advisory board fees for AbbVie, Celgene, Ferring, Janssen, BMS, Pfizer, Takeda, Gilead-Galapagos, Arena and Elli Lilly; and consultancy fees from AbbVie.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The STORI study was registered with ClinicalTrials.gov (NCT00571337), and approved by the Ethics Committee of the Saint-Louis Hospital (CPP 2005/2014) and the AFSSAPS (0809/ALV/EG05). The SPARE study was registered with ClinicalTrials.gov (NCT02177071) and with EU Clinical Trials Register (EUDRACT 2014-002311-41), and approved by the appropriate institutional review boards or ethics committees of each country. The STORI and SPARE studies were executed in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and applicable local regulations. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. All the data (clinical, selected reaction monitoring (SRM) and proximity extension assay) supporting our findings are available at https://panoramaweb.org/7PNxFY.url. For the STORI study, SRM raw data are available at https://panoramaweb.org/atQBcH.url (dataset identifier: PXD019434). For the SPARE study, SRM raw data are available at https://panoramaweb.org/7PNxFY.url (dataset identifier: PXD051233). The Python codes used for data analysis are available at https://github.com/vahuynh/STORI_SPARE.

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