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Intestinal tissue levels of anti-TNF alpha, antibodies, and cytokines in paediatric Crohn disease

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The aim was to explore factors associated with intestinal tissue levels of anti-TNF alpha (anti-TNF), anti-TNF antibodies, and cytokines in pediatric patients with Crohn Disease (CD). In a prospective exploratory study of CD patients undergoing ileocecal resection or colonoscopy between 6/2020 and 1/2023, we analysed tissue levels of anti-TNF, anti-TNF antibodies, and cytokines (TNF-α, IL-17, IL-1β, IFN-γ) from intestinal biopsies. Mixed-effects regression models, adjusted for potential confounders, were used. Data from 27 CD patients (18 females, 66.7%) were analysed. Fourteen (52%) received adalimumab (ADA) and thirteen received infliximab (IFX), with a median therapy duration of 17 (IQR 4.5–41.5) months. Higher levels of free anti-TNF were found in macroscopically inflamed tissue compared to non-inflamed tissue (β = 3.42, 95% CI 1.05–6.10). No significant association was found between serum and tissue anti-TNF levels (β= −0.06, 95% CI −0.70–0.58). Patients treated longer with anti-TNF had increased IL-17 levels (β = 0.19, 95% CI 0.05–0.33), independent of disease duration and age. IFN-γ levels were linked with both follow-up duration and anti-TNF length. Our study shows significantly higher free drug levels in inflamed tissue. Long-term anti-TNF treatment has been linked to increased IL-17 levels, suggesting a possible impact on the cytokine response pathway. We did not observe a relationship between serum and tissue anti-TNF levels.

Keywords Inflammatory bowel disease, Paediatrics, Biologics, Crohn disease

Crohn disease (CD) is a chronic immune-mediated condition with increasing incidence worldwide[1]. Tumour necrosis factor alpha (TNF-α) plays a key role in the inflammatory response and is a major target for modern CD therapies[2,3]. Significant advancements include biologic therapies, particularly anti-TNF agents. These biologics, adalimumab (ADA) and infliximab (IFX), are recombinant IgG1 monoclonal antibodies[4,5] that bind and neutralise human TNF-α[6].

Therapeutic drug monitoring (TDM) in serum is used to adjust anti-TNF treatments[7,8], yet population-based registries show that 25% of children lose response within three years[9]. While pharmacokinetics explains some failures, a significant portion remains unexplained[10]. Attempts to elucidate the reasons behind the failure of biological treatments in certain subgroups by directly measuring biologic levels in intestinal tissue have been documented by researchers in the USA[11], Japan[12], and Israel[13]. This method, however, has not yet been applied to paediatric patients. Moreover, data on this subject are generally scarce, underscoring a notable gap in our knowledge in this area. Exploring tissue-level pharmacokinetics may help uncover reasons for treatment failure in pediatric patients and inform more effective therapeutic strategies. To better understand the effect of treatment outcome, we complemented this analysis by monitoring the levels of major pro-inflammatory cytokines involved in the pathogenesis of IBD.

This study aimed to explore factors associated with intestinal tissue levels of anti-TNF and anti-TNF antibodies, as well as their relationship with selected tissue cytokines in pediatric CD patients.

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Materials and methods

Study design

This single-centre, cross-sectional exploratory study investigated relationships between tissue levels of anti-TNF drugs, anti-TNF antibodies, and cytokines. Intestinal tissue biopsies were collected during routine endoscopy or ileocecal resection.

Population

Pediatric CD patients (≤ 19 years) undergoing ileocolonoscopy or ileocecal resection (ICR) at Motol University Hospital and treated with anti-TNF were included (Supplementary Figure S1).

All patients were diagnosed with CD based on the revised Porto criteria[14] and were treated according to currently valid European Crohn's and Colitis Organisation (ECCO) and European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines[15,16]. Patients were referred for the procedure by the treating gastroenterologist or surgeon as part of standard clinical care, independent of this study. Data were prospectively recorded for each patient. At the same time, both peripheral blood and intestinal biopsies were collected.

The induction phase, considered standard for all patients, involved the administration of adalimumab with dosages of 160–80–40 mg (patients > 40 kg) or 80–40–20 mg (patients < 40 kg), given every 2 weeks. Infliximab was administered at a dosage of 5 mg/kg at weeks 0, 2, and 6. Patients were treated with biologic therapy up until the time of surgery, with no specific washout period for anti-TNF.

Intestinal biopsy sampling and processing

Biopsy sampling

Intestinal samples were collected using biopsy forceps during the colonoscopy from inflamed and non-inflamed tissue in the terminal ileum and ascending colon. A senior gastroenterologist was always present during the assessment of macroscopic inflammation. If no macroscopic signs of inflammation were present, tissue samples were collected solely from non-inflamed areas. The same protocol was followed during ileocecal resection (ICR), utilizing a 3 mm biopsy punch (Miltex®) to extract an equivalent amount of tissue.

Sample processing

Biopsy weight was recorded prior to cultivation. Subsequently, the biopsy was cultivated in pre-warmed Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich; Cat# R0883), containing 10% fetal bovine serum (FBS) (HyClone, Cytiva), 1% antibiotic-antimycotic solution (Sigma-Aldrich; Cat# P 0781), and 1% L-glutamine solution (Sigma-Aldrich; Cat# 1.00289) and placed into a humidified incubator (37 °C, 5% CO2) for 48 h. After incubation, the supernatant was collected, aliquoted, and frozen (− 20 °C) until the analysis. In serum, the level of free IFX, free ADA free antibodies to IFX and to ADA were measured directly.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of free IFX, free ADA, free antibodies to IFX, and to ADA were measured in serum and tissue culture supernatants using commercial ELISA kits according to the manufacturer's instructions. The ELISA kit to determine free IFX and free ADA are coated with highly specific antibodies against IFX or ADA. These kits demonstrated a sensitivity of 3 ng/mL with a spike recovery of > 95%, ensuring no cross-reactivity with other therapeutic monoclonal antibodies. The commercial kits for quantifying free IFX or ADA and for detecting antibodies to IFX or ADA are validated for both research and diagnostic applications, making them suitable for sensitive therapeutic drug monitoring (TDM). The assay for detecting antibodies to IFX and ADA is drug-sensitive. The levels of Tumour Necrosis Factor alpha (TNF-α), Interleukin-17 (IL-17), Interleukin-1 beta (IL-1β), and Interferon-gamma (IFN-γ) was quantified by ELISA in tissue culture supernatants according to the manufacturer's instructions (Supplementary Table S1). Absorbance was measured at 450 nm and 650 nm by spectrophotometer (Multiskan Ascent Plate Reader 96/384, MTX Lab Systems).

All levels were normalised to tissue weight and expressed as concentration per mg of tissue.

Covariates

The data included patients' age, sex, body mass index, wPCDAI (< 10: remission)[17], administration of medication (corticosteroids or immunomodulators, and biologics), laboratory parameters (C-reactive protein [CRP] and faecal Calprotectin [f-CPT]), disease duration, and disease behaviour according to the Paris classification[18], indication for endoscopy and surgery, timing of the procedure, type of procedure performed, 30-day postoperative complications according to the Clavien–Dindo classification (CDc)[19], and follow-up. Endoscopic findings were assessed according to the Simple endoscopic score for CD (SES-CD; 0–2: remission)[20] or the modified Rutgeerts scoring system for patients undergoing ICR (Ri0–Ri1: remission)[21,22]. These variables were incorporated as covariates in multivariate analyses to adjust for potential confounders.

Statistical analysis

Statistical analyses were performed using R software (version 4.3.0; www.r-project.org). Continuous variables were summarised as medians with interquartile ranges (IQR), categorical variables as counts and percentages. The dataset was complete with no missing values.

To account for the multiple samples obtained from different tissues of the same patient, we employed a linear mixed-effects regression in all models. Initially, associations between individual measured tissue values and explanatory variables were tested using a mixed model adjusted only for individuals as a random effect. Variables included tissue localization, presence of inflammation, sample collection methodology, type of anti-TNF

medication, serum levels of both anti-TNF and anti-TNF antibodies, disease duration, duration of anti-TNF administration, and age. Associations were further evaluated using a mixed multiple linear regression model, adjusted for additional factors, which are detailed in the text or corresponding tables. Graphical assessments were performed for individual relationships and supplemented with sensitivity analyses where appropriate.

Statistical significance was set at $p < 0.05$. Results are reported with a 95% confidence interval (CI) to provide precision estimates. All graphical representations of data were created using the "ggplot2" package in R.

Ethical commission

This study was approved by the Ethics Committee of the University Hospital Motol (Reference No.: EK-1263.1.4/19). The research was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents.

Results

We included 95 tissue samples (Supplementary Figure S2; Supplementary Table S2) from 27 CD paediatric patients (18 females, 66.7%), of whom 16 (59.3%) underwent endoscopy. No differences were observed between endoscopic and surgical sampling methods (Supplementary Table S3), except for IFN-γ. Patient characteristics and related data are detailed in Table 1 and Supplementary Table S4. None of the patients were receiving corticosteroid therapy, and thirteen patients (48.1%) were on intensified anti-TNF therapy (8 IFX, 5 ADA). For further details on pharmacotherapy, refer to Supplementary Table S5. The indications for colonoscopy and surgery are summarized in Supplementary Table S6.

Tissue anti-TNF

First, we measured serum and tissue anti-TNF drug concentration. Median serum levels of ADA and IFX were 15 µg/mL (IQR: 15, 15.2) and 15 µg/mL (IQR: 12, 17.5), respectively. The median tissue level of ADA in non-inflamed tissue was 2.89 (IQR: 0.07, 9.05) ng/ml per mg of tissue and in inflamed tissue 7.10 (IQR: 1.06, 19.9) ng/ml per mg of tissue, and the median IFX level in non-inflamed and inflamed tissue was 1.60 (IQR: 0.00, 5.72)

Variable	Overall, $n = 27$	IFX, $n = 13$ (48.1%)	ADA, $n = 14$ (51.9%)
Sex; Female (%)	18 (66.7)	9 (69.2)	9 (64.3)
Age at diagnosis, years (median, Q1, Q3)	12.3 (10.2, 14.3)	12.7 (9.6, 15.0)	12.0 (10.1, 13.1)
Age at surgery, years (median, Q1, Q3)	-	16.3 (13.7, 17.1)	17.5 (14.7, 18.4)
Age at endoscopy, years (median, Q1, Q3)	-	16.1 (12.0, 18.5)	18.5 (16.5, 19.0)
Disease duration, months (median, Q1, Q3)	50.0 (23.0, 69.0)	36.0 (16.5, 50.5)	66.0 (44.5, 82.5)
Time to initiation of anti-TNF therapy (months) (median, Q1, Q3)	17.5 (2.0, 36.0)	17.0 (2.0, 40.0)	21.0 (5.0, 36.5)
Duration of the biologic therapy until the procedure (months) (median, Q1, Q3)	17.0 (4.0, 43.0)	13.0 (4.0, 22.0)	36.0 (3.75, 68.0)
Number of days from last anti-TNF dose to procedure (days) (median, Q1, Q3)	12.0 (6.0, 23.0)	23.0 (14.0, 37.5)	7.0 (4.5, 10.5)
Endoscopy group (%)	16 (59.3)	7 (53.8)	9 (64.3)
Surgery group (%)	11 (40.7)	6 (46.2)	5 (35.7)
Disease behaviour (%)			
B1 (non-stricturing / non-penetrating)	14 (51.9)	6 (46.2)	8 (57.1)
B2 (stricturing)	8 (28.6)	4 (30.8)	4 (28.6)
B3 (penetrating)	5 (18.5)	3 (23.1)	2 (14.3)
Perianal form	4 (14.8)	2 (15.4)	2 (14.3)
Disease activity (wPCDAI) (%)			
Remission (< 10)	18 (66.7)	8 (61.5)	10 (71.4)
Mild (10–27,5)	7 (25.9)	4 (30.8)	3 (21.4)
Moderate (30–37,5)	2 (7.4)	1 (7.7)	1 (7.1)
Severe (> = 40)	0 (0)	0 (0)	0 (0)
CRP [mg/l]	1.1 (0.5, 7.4)	1.1 (0.5, 6.4)	1.9 (0.5, 13.23)
f-CPT [ug/g]	844.0 (145.0, 1483)	862.0 (448.5, 1782)	597.0 (50.75, 1358)
Albumin levels at time of collection [g/l]	47.5 (43.35, 48.05)	47.5 (43.35, 48.9)	47.45 (42.45, 48.2)
Endoscopic remission in colonoscopy (SES-CD, Rutgeerts score) (%)			
Yes	7 (43.8)	2 (28.6)	5 (55.6)
No	9 (56.3)	5 (71.4)	4 (44.4)

Table 1. Patients' characteristics. BMI, body mass index; IQR, interquartile range; TNF-α, tumour necrosis factor α; SES-CD, simple endoscopic score; CRP, C-reactive protein, f-CPT faecal calprotectin, wPCDAI, weighted Pediatric Crohn disease activity index; Disease behaviour according to Paris classification; SES-CD remission − 0–2; Rutgeerts score remission < Ri2.

and 3.12 (IQR: 1.69, 9.70) ng/ml per mg of tissue, respectively. Serum and tissue levels stratified by endoscopic activity are detailed in Supplementary Table S7. There was no association between serum and tissue anti-TNF (β= −0.06, 95% CI −0.70–0.58).

Next, we investigated the possible association between the level of anti-TNF in the tissue without inflammation and with macroscopic inflammation. Paired samples from 22 patients were available for analysis. We observed that tissue drug levels were higher in samples from macroscopically inflamed tissue than in non-inflamed tissue (Fig. 1), even after adjusting for factors such as type of anti-TNF treatment, disease localization, sampling method, TNF-α, and individual variations (β = 3.42, 95% CI 1.05–6.10) (Table 2). The ratio of anti-TNF level in tissue to serum was higher in the inflamed tissue (β = 0.21, 95% CI 0.04–0.38) than in non-inflamed tissue (Supplementary Table S8).

Despite appropriate serum levels of anti-TNF (>=5 ug/mL), five (19%) patients showed no detectable free anti-TNF levels in the ileal tissue. Of these, three (23.1%) were treated with IFX.

Tissue anti-TNF antibodies

We measured serum and tissue anti-TNF antibodies, their relationships, and effects on tissue drug levels. Median serum antibody levels against IFX and ADA were 0.2 AU/mL (IQR: 0, 0.2) and 0.8 AU/mL (IQR: 0, 1.1). Median tissue levels of antibodies against ADA were 0 (IQR: 0.0, 0.14) in non-inflammatory and 0.01 (IQR: 0.0, 0.31) AU/ml per mg in inflammatory tissue. For IFX, median tissue levels were 0 (IQR: 0.0, 0.01) in non-inflamed and 0 (IQR: 0.0, 0.06) in inflamed tissue. When analyzing only non-zero values, the median serum antibody levels were 0.2 AU/mL (IQR: 0.1, 1.25) for IFX and 1.1 AU/mL (IQR: 0.7, 1.55) for ADA. Median tissue levels were 0.05 AU/mL per mg (IQR: 0.03, 0.08) for IFX and 0.12 AU/mL per mg (IQR: 0.05, 0.87) for ADA in non-inflammatory tissue, and 0.08 AU/mL per mg (IQR: 0.03, 0.83) for IFX and 0.28 AU/mL per mg (IQR: 0.05, 0.84) for ADA in inflammatory tissue. No significant association was observed between serum and tissue anti-TNF antibodies (β = 0.5, 95% CI −0.29–1.23)., nor between antibody levels in non-inflamed and inflamed tissues (β = 0.60, 95% CI −0.20–1.42).

In the primary analysis, we found that higher antibody levels were associated with lower tissue levels of free anti-TNF (β= −0.76, 95% CI −1.37 – −0.13). Following graphical analysis, we opted to test this relationship within a sensitivity analysis after excluding one outlier value. In this analysis, we did not find the association (β = 3.68, 95% CI −1.17–8.75).

We identified serum anti-TNF antibodies above 0 AU/mL in 11 (40.74%) patients (six treated with IFX, five with ADA). Among these, four (36.4%) had detectable antibodies in non-inflamed ileal tissue (three IFX, one ADA), and six (54.5%) in inflamed ileal tissue (three IFX, three ADA). Among the 16 (59.26%) patients without detectable serum antibodies, tissue antibodies were present in six (37.5%) non-inflamed (one IFX, five ADA) and five (31.2%) inflamed ileal tissues (two IFX, three ADA).

Tissue cytokines

Tumour necrosis factor-alpha

Our analysis revealed that TNF-α levels in tissue were associated with serum anti-TNF levels and patient age. In the adjusted model (Supplementary Table S9), TNF-α levels were significantly associated with the type of anti-TNF therapy, with higher levels observed in IFX-treated patients (β = 47.57, 95% CI 21.70–73.37). However, no significant association was identified between TNF-α levels and the presence of tissue inflammation (β = 1.18, 95% CI −0.09–2.50).

Interleukin-17

Tissue IL-17 levels were associated with the duration of anti-TNF therapy (β = 0.16, 95% CI 0.02–0.30), patient age (β= −2.02, 95% CI −3.34 – −0.71), and TNF-α levels (β = 1.00, 95% CI 0.75–1.25).

In the adjusted model, IL-17 levels increased with the duration of anti-TNF treatment (β = 0.19, 95% CI 0.05–0.33) per month, independent of disease duration. This association was stronger in inflamed tissues (β = 3.44, 95% CI 1.05–5.93) and in patients with detectable tissue anti-TNF antibodies (β= −6.37, 95% CI −11.68 – −1.03) (Supplementary Table S10).

Interleukin-1 beta

Tissue IL-1β levels were associated with the presence of inflammation (β = 81.65, 95% CI 12.61–153.52), TNF-α levels (β = 12.19, 95% CI 7.23–17.15), and patient age (β= −24.26, 95% CI −40.94 – −8.00).

IL-1β levels remained significantly higher in inflamed tissue compared to non-inflamed tissue, even after adjusting for anti-TNF treatment type, tissue localization, and age (β = 69.64, 95% CI 3.01–138.92) (Supplementary Table S11).

Interferon-gamma

Tissue IFN-γ levels were associated with the method of collection (β= −23.85, 95% CI −46.00 – −1.58), the duration of anti-TNF therapy (β = 0.59, 95% CI 0.20–0.97), and the disease duration (β = 5.64, 95% CI 1.80–9.46).

In the adjusted model, no significant difference in IFN-γ levels was observed between inflamed and non-inflamed tissues. However, IFN-γ levels were positively associated with disease duration (β = 5.80, 95% CI 1.69–9.90) and the duration of anti-TNF therapy (β = 0.57, 95% CI 0.18–0.96) (Supplementary Table S12).

Discussion

To the best of our knowledge, this is the first study to explore tissue-level pharmacokinetics of anti-TNF drugs in paediatric CD patients, while also examining inflammation-associated cytokines. We found significantly higher

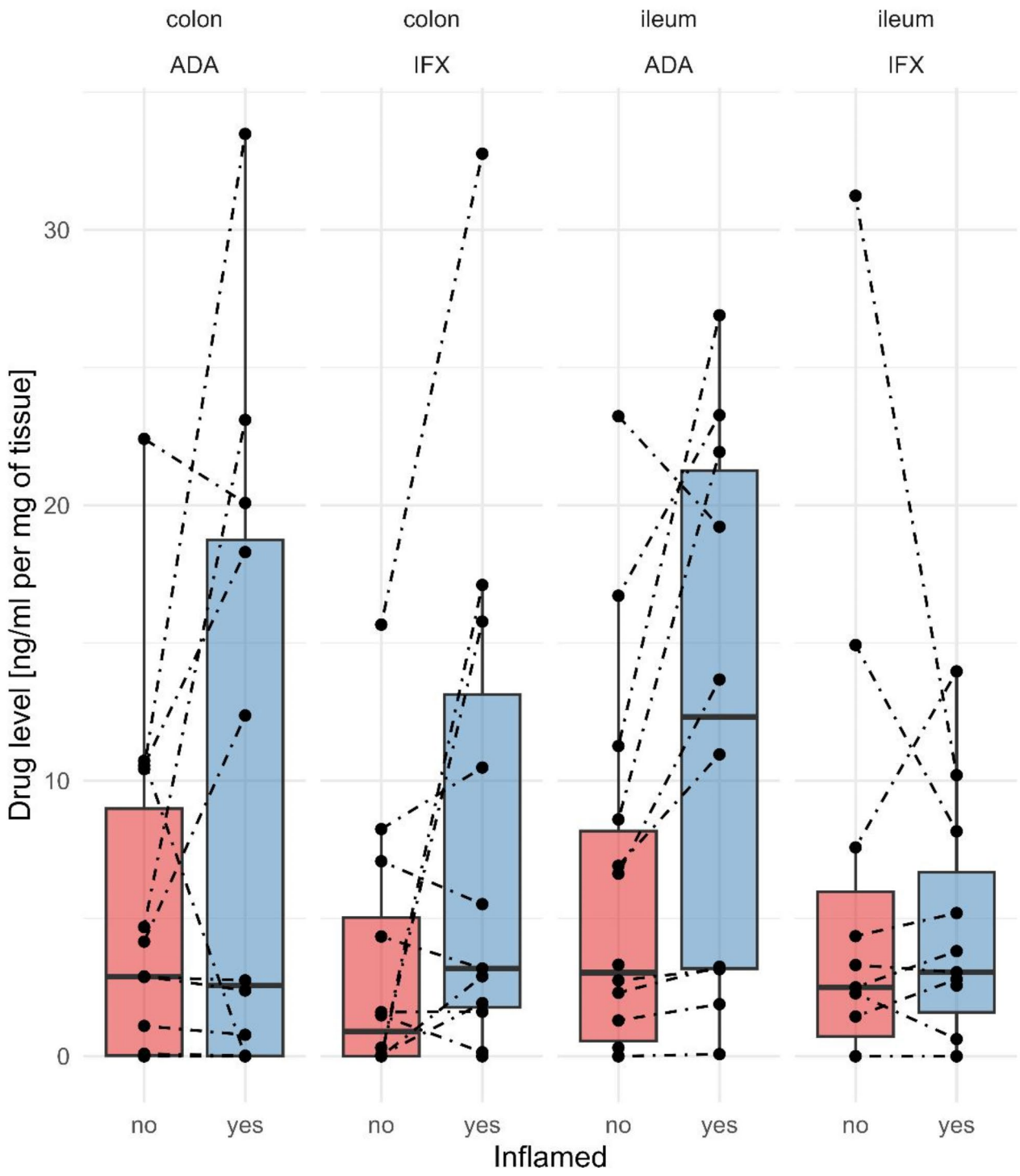

Fig. 1. Tissue levels in inflamed and non-inflamed tissue according to anti-TNF type and localization. Observations are shown separated by sampling location and type of anti-TNF therapy. The dashed line connects points in inflamed and non-inflamed tissue of one patient. ADA, adalimumab; IFX, infliximab; Inflammation - no/yes.

anti-TNF levels in inflamed tissue compared to non-inflamed tissue; however, no association was observed between tissue levels and measured serum levels.

Our findings align with previous studies that observed significantly higher levels of free anti-TNF in the mucosa with macroscopic inflammation[11–13]. These studies suggest that inflamed tissue produces excessive TNF-α, which fuels inflammation and attracts anti-TNF medications[11]. This supports the hypothesis that

	β	95% CI	*p* value
Tissue inflammation (yes/no)	3.42	(1.05–6.10)	0.01
Type of anti-TNF (infliximab/adalimumab)	-1.75	(-7.74-4.30)	0.62
Localization of biopsy (ileum/colon)	0.39	(-1.93-2.79)	0.75
Method of collection (surgery/endoscopy)	2.38	(-3.68-8.34)	0.50
Actual age (years)	0.95	(-0.33-2.20)	0.19
Length of follow-up (years)	-1.08	(-2.48-0.34)	0.20
Length of anti-TNF treatment (years)	0.09	(-0.06-0.23)	0.32
Levels of TNF-α (pg/ml per mg of tissue)	0.07	(-0.28-0.39)	0.68

Table 2. Association between free anti-TNF tissue levels and pre-defined variables in multivariable mixed model. The association was assessed using a multivariable mixed model, which was additionally adjusted for patients in a random part of the model. TNF-α; tumour necrosis factor alpha; anti-TNF, anti-tumour necrosis factor α.

increased TNF-α levels lead to more anti-TNF binding in inflamed tissue, but anti-TNF alone may not sufficiently neutralise TNF-α, resulting in a lower anti-TNF/TNF-α ratio; inflammatory tissue acts as a reservoir for anti-TNF[11].

However, it is important to note that these previous findings were derived from tissue samples from both CD and ulcerative colitis patients[11,13], where inflammation mediation may vary[23,24]. Additionally, one study had a relatively low number of biopsies from inflamed tissue (17/60)[11]. Other studies[12] reported lower TNF-α levels in inflamed tissue compared to non-inflamed tissue, suggesting alternative explanations like tissue destruction increasing permeability to anti-TNF drugs[25] or anti-TNF binding with TNF-α in complexes, making it unmeasurable[13]. Future measurements of anti-TNF in stool could further elucidate reasons for anti-TNF loss.

Interestingly, we observed higher TNF-α levels in IFX-treated patients compared to those receiving ADA, despite comparable tissue anti-TNF levels. The exact reason for this observation remains unknown, but possible explanations include IFX's stabilizing effect on TNF-α through the formation of larger complexes, which may reduce clearance and prolong TNF-α presence in inflamed tissue[4]; its stronger Fc-mediated effector functions, such as complement activation and cell apoptosis, which could induce compensatory TNF-α production[26]; differences in neutralizing mechanisms, where IFX binds more strongly to membrane-bound TNF-α, potentially promoting its localized persistence[27,28]; IFX's direct interaction with intestinal mucosa, which sustains cytokine production as part of its mucosal healing effects[29]; and pharmacokinetic differences, with IFX showing slower clearance and prolonged tissue retention compared to ADA[30]. Additionally, IFX's higher immunogenic potential may lead to anti-drug antibody formation, indirectly amplifying TNF-α levels by reducing drug efficacy[26,31]. Differences in administration routes (intravenous for IFX and subcutaneous for ADA) may also contribute to variability in local tissue dynamics of TNF-α[32].

Studies in adults have shown that the relationship between serum levels and free drugs is best found in non-inflamed intestinal tissue. However, our pediatric study found no such association. Yarur et al.[11] observed an association between serum levels and non-inflamed tissue levels for IFX. A Japanese study[12] from 2017 reported an association only after dividing serum drug levels into high and low categories. Bar-Yoseph et al.[13] found associations between serum levels and tissue levels of free ADA and IFX. Our patient group was comparable in size to previous studies; hence, this alone cannot be deemed a comprehensive explanation. It prompts speculation regarding whether variances in the pharmacokinetics of monoclonal antibodies in paediatric patients may elucidate this phenomenon[33,34].

In our study, despite serum drug levels > 5 µg/mL, five (19%) patients had no measurable anti-TNF in non-inflammatory ileal tissue. Similar findings were reported in previous studies[11], showing better long-term outcomes in adults with high serum and non-inflammatory tissue levels[12]. These observations raise the possibility that measuring only serum anti-TNF levels might not fully capture therapeutic dynamics in certain cases. Incorporating tissue anti-TNF measurements during endoscopy could potentially complement serum assessments and provide additional beneficial insights into therapeutic effectiveness. To address this, further prospective studies comparing patients managed by serum levels alone versus those also monitored by tissue levels are needed.

We monitored tissue activity in response to two anti-TNF therapies: one humanised and one chimeric, aiming to identify differences in antibody responses. No such distinctions were observed. Our analysis also found no significant correlation between serum and tissue anti-drug antibody levels, regardless of inflammation. Initially, higher serum antibody levels seemed linked to lower tissue anti-TNF levels, but this correlation disappeared after accounting for outliers.

Our results showed that the presence of antibodies in one compartment did not predict their presence in another. Additionally, high median serum levels of anti-TNF were observed, with nearly half the patients on concomitant immunosuppressants, which, among other factors, can influence anti-TNF immunogenicity[16,35–38]. However, due to the small number and heterogeneity of antibody observations in both serum and tissue, it's challenging to determine if measuring tissue antibodies offers a significant advantage. A more conclusive assessment would require simultaneous monitoring of serum and tissue levels to see if predictive accuracy improves. Moreover, no existing research has specifically evaluated tissue anti-drug antibodies[11–13].

We also monitored the level of the four major pro-inflammatory cytokines TNF-α, IL-17, IL-1β and IFN-γ in tissue culture supernatants. While other studies have shown elevated levels of these cytokines in serum of IBD patients, our approach has allowed us to gain unique insight into the inflammatory response in tissue with or without inflammation during the anti-TNF therapy. These cytokines were selected because of their implication in the IBD pathogenesis. IFN-γ is one of the major pathogenetic factors in IBD and can severely disrupt the intestinal barrier and is thus associated with increased inflammation and progression of IBD[39]. IL-1β promotes chronic intestinal inflammation and tissue damage in IBD through recruitment of immune cells, promotion of the Th17 response of CD4 + T cells and activation of innate lymphoid cells (ILCs) in the intestine[40–42]. The balance between Th17 and regulatory T cells is crucial for maintaining intestinal homeostasis, in inflammatory bowel diseases Th17 cells expand and secrete pro-inflammatory cytokines (including IL-17). IL-17 has strong pro-inflammatory activity and has been described to be significantly elevated in active colitis and CD[43,44].

From this analysis, of particular interest is our observation of IL-17 accumulation with prolonged anti-TNF therapy, especially in inflamed tissues with higher anti-drug antibody prevalence. IFN-γ also accumulated, correlating with disease duration and anti-TNF treatment length. These findings highlight the pivotal role of the IL-23/IL-17[45] axis in CD. IL-23[46,47], synthesised by antigen-presenting cells, promotes the expansion of Th17 cells, a primary source of IL-17, driving the inflammation cascade.

These observations align with the concept that some patients on anti-TNF therapy may develop resistant IL-17 and IFN-γ-producing cell clones[46,48,49], supporting the potential use of anti-IL-17 drugs. However, studies on anti-IL-17 therapy have shown mixed results, with some reporting condition exacerbation due to IL-17's role in maintaining intestinal barrier integrity[50,51]. This supports the rationale for considering anti-IL-23 therapy, which shares the IL-17 pathway but without the adverse effects seen with anti-IL-17 therapies[52].

Strengths and limitations

Our prospectively collected data reduced the risk of recall bias. The breadth of the data allowed us to adjust for several confounders.

The sample size was relatively small but comparable to previous studies[11,12]. Multiple sampling sites per patient resulted in one of the highest numbers of samples analyzed in published studies. We employed appropriate statistical methods to account for multiple samples from single patients.

The macroscopic presence of inflammation was assessed by the examining physician, but senior gastroenterologists were always present, making it unlikely to affect results. We compared sampling methods during ileocecal resection and endoscopy, which have slightly different mechanisms. Statistical analyses showed no significant differences between sampling types, indicating that routine endoscopic tissue biopsies are sufficient and reliable.

The exploratory nature of this study requires caution when generalizing the findings, and the cross-sectional design limits conclusions about anti-TNF failure mechanisms over time. However, given the limited data on direct intestinal tissue measurements of anti-TNF in children, this approach was necessary and provides a foundation for future research.

Conclusion

In conclusion, our study offers novel insights into tissue levels of anti-TNF therapies in children with CD. We found a pronounced concentration gradient of anti-TNF in intestinal tissues based on inflammation. While serum anti-TNF antibodies were detectable, tissue antibodies were negligible, indicating a potential dissociation between systemic and local immune responses. Prolonged anti-TNF therapy correlated with increased tissue levels of IL-17, regardless of disease duration or patient age, suggesting an influence on the inflammatory cytokine pathway. Future larger-scale studies should evaluate serum, tissue, and stool levels of anti-TNF and measure IL-23 to better understand anti-TNF therapy mechanisms in intestinal tissue.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
V.D. conceived the study idea and designed the study. S.C. conducted laboratory examinations and contributed to the study design. V.D., T.L., and K.Z. were involved in patient recruitment and data collection. T.L., O.H., and V.D. analysed and interpreted the data. V.D., O.H., and T.L. drafted the manuscript. All authors critically revised the manuscript for important intellectual content and approved the final version for submission.

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Declarations

Competing interests
The authors declare no competing interests.

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