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Expression patterns of TNF , MAdCAM1 and STAT3 in intestinal and skin manifestations of inflammatory bowel disease

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Abstract: Background: Pathogenesis of cutaneous extraintestinal manifestations (EIM) in inflammatory bowel disease (IBD) remains elusive. Efficacy of anti-TNF agents suggests TNF-dependent mechanisms. The role of other biologics such as anti-integrins or JAK-inhibitors is not yet clear. Methods: We performed immunohistochemistry for TNF , NF B, STAT1/STAT3, MAdCAM1, CD20/68, caspase 3/9, IFN , Hsp-27/70 on 240 intestinal (55 controls, 185 IBD) and 64 skin biopsies (11 controls, 18 Erythema nodosum (EN), 13 Pyoderma gangenosum (PG), 22 psoriasis). A semiquantitative score (0-100%) was used for evaluation. Results: TNF was upregulated in intestinal biopsies from active Crohn's disease (CD) vs. controls (36.2 vs. 12.1, p<0.001), but not ulcerative colitis (UC: 17.9). NF B however was upregulated in intestinal biopsies from both active CD and UC (43.2 and 34.5 vs. 21.8, p<0.001 and p=0.017). TNF and NF B were overexpressed in skin biopsies from EN, PG and psoriasis. No MAdCAM1 overexpression was seen in skin tissues, while it was upregulated in active UC vs. controls (57.5 vs. 35.4, p=0.003). STAT3 was overexpressed in the intestinal mucosa of active and non-active IBD, while a similar upregulation was seen in skin biopsies from EN (84.7 vs. 22.3, p<0.001) and PG (60.5 vs. 22.3, p=0.011), but not in psoriasis. Caspase 3 and CD68 overexpression in skin biopsies distinguished EN/PG from psoriasis and controls. Conclusions: Upregulation of TNF /NF B in EN and PG is compatible with the efficacy of anti-TNF in EIM management. Data on overexpressed STAT3, but not MAdCAM1 support a rationale for JAK-inhibitors in EN and PG, while questioning the role of vedolizumab.

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Expression Patterns of TNFα, MAdCAM1 and STAT3 in Intestinal and Skin Manifestations of Inflammatory Bowel Disease

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ABSTRACT

Background: Pathogenesis of cutaneous extraintestinal manifestations (EIM) in inflammatory bowel disease (IBD) remains elusive. Efficacy of anti-TNF agents suggests TNF-dependent mechanisms. The role of other biologics such as anti-integrins or JAK-inhibitors is not yet clear.

Methods: We performed immunohistochemistry for TNFα, NFκB, STAT1/STAT3, MAdCAM1, CD20/68, caspase 3/9, IFNγ, Hsp-27/70 on 240 intestinal (55 controls, 185 IBD) and 64 skin biopsies (11 controls, 18 Erythema nodosum (EN), 13 Pyoderma gangenosum (PG), 22 psoriasis). A semiquantitative score (0-100%) was used for evaluation.

Results: TNFα was upregulated in intestinal biopsies from active Crohn`s disease (CD) vs. controls (36.2 vs. 12.1, p<0.001), but not ulcerative colitis (UC: 17.9). NFκB however was upregulated in intestinal biopsies from both active CD and UC (43.2 and 34.5 vs. 21.8, p<0.001 and p=0.017). TNFα and NFκB were overexpressed in skin biopsies from EN, PG and psoriasis. No MAdCAM1 overexpression was seen in skin tissues, while it was upregulated in active UC vs. controls (57.5 vs. 35.4, p=0.003). STAT3 was overexpressed in the intestinal mucosa of active and non-active IBD, while a similar upregulation was seen in skin biopsies from EN (84.7 vs. 22.3, p<0.001) and PG (60.5 vs. 22.3, p=0.011), but not in psoriasis. Caspase 3 and CD68 overexpression in skin biopsies distinguished EN/PG from psoriasis and controls.

Conclusions: Upregulation of TNFα/NFκB in EN and PG is compatible with the efficacy of anti-TNF in EIM management. Data on overexpressed STAT3, but not MAdCAM1 support a rationale for JAK-inhibitors in EN and PG, while questioning the role of vedolizumab.

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Keywords: extraintestinal manifestations, anti-TNF, anti-integrins, JAK-inhibitor, immunohistochemistry, microarray

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INTRODUCTION

Inflammatory bowel disease (IBD) with the two main subtypes Crohn`s disease (CD) and ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract. The etiopathogenesis of IBD is incompletely understood, although it is considered being a multifactorial disease, which arises from a complex interplay between genetic, environmental and immunological factors with an abnormal host immune response to environmental stimuli. (1) Different cytokines and cell interaction proteins have been identified as key players in IBD pathogenesis such as the TNFα-NFκB axis, the JAK-STAT pathway and the integrin-vascular adhesion molecule interaction. Several already approved drugs or investigational agents take advantage of these pathomechanisms. Anti-TNF agents have been successfully used for more than a decade, while anti-integrins such as vedolizumab have been introduced into clinical practice only recently. (2, 3) The latter target the gut-specific interaction between integrin α4β7 on leukocytes and the adhesion molecule MAdCAM1 on endothelial cells in the intestine, thereby blocking leukocyte adhesion and migration to the site of inflammation. Few if any systemic side effects are observed. (2, 3) Tofacitinib represents a first class oral agent inhibiting the Janus kinase (JAK) family of proteins, which are important mediators in inflammation. Blocking JAK downregulates proinflammatory cytokines such as interleukin (IL) 2, 4, 7, 9, 15 and 21 through the JAK-STAT pathway. (4) Efficacy has been reported for UC, but not for CD, while more specific JAK inhibitors such as filgotinib may also be efficacious in the latter indication. (5-7) Approval process for tofacitinib`s use in UC patients has been recently initiated by the US Food and Drug Administration (FDA).

Extraintestinal manifestations (EIM) of IBD are common with a frequency ranging from 6 to 47%. (8-15) Besides arthritis and stomatitis, cutaneous manifestations are among the most prevalent EIM. (15) IBD skin lesions mainly include erythema nodosum (EN) and pyoderma gangrenosum (PG). While EN usually parallels intestinal disease activity, PG may or may not be associated with intestinal

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disease. The pathomechanisms of EN and PG remain elusive, although efficacy of anti-TNF treatment suggests TNF-dependent mechanisms. Limited data from immunohistochemical evaluations and protein analyses from tissue lysates have demonstrated upregulation of several inflammatory cytokines such as IL 8, 17, 1β and TNFα in PG. (16, 17) Evidence for the efficacy of anti-TNF in EIM management is evolving. Recently, a thorough meta-analysis conducted by Peyrin-Biroulet and colleagues has shown response rates between 69-100% for PG and reduction of EN prevalence from 2.4 to 0.4% with anti-TNF treatment. (18, 19) If other biological agents are efficacious in the treatment of EN or PG has yet to be determined. Studies evaluating the role of vedolizumab are under way. It is yet unclear if the gut-selective mode of action limits its efficacy to the intestine or if indirect beneficial effects via decrease of intestinal inflammatory load might be relevant. No data is available so far regarding the influence of JAK inhibitors on EIM, although tofacitinib`s efficacy has been shown for other auto-inflammatory disorders such as rheumatoid arthritis and psoriasis. (20, 21)

We herein investigated different proteins involved in intestinal and extraintestinal IBD manifestations aiming at elucidating similarities and differences in IBD, cutaneous EIM and psoriasis pathophysiology with a special focus on possible therapeutic implications such as involvement of the TNFα-NFκB and JAK-STAT pathway as well as the integrin-MAdCAM interaction.

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METHODS

Study design

In this observational single-center study, we prospectively collected intestinal and cutaneous tissue samples from healthy individuals and patients with IBD, cutaneous EIM and psoriasis. Sample collection was conducted between 2004 and 2011. All patients had previously given their written informed consent for tissue collection and review of patient charts. All data were anonymized. The study was supported by the Swiss National Science Foundation and was approved by the local ethics committee of the University Hospital Zurich (KEK-ZH-837).

Patient and data collection

All patients analyzed in this study were treated for IBD and/or inflammatory skin disease such as EN, PG or psoriasis at the University Hospital Zurich, Switzerland. Diagnosis of UC and CD had been previously established according to the current ECCO guidelines. (22, 23) Inflammatory skin lesions had all been diagnosed by a dermatologist. Intestinal samples from healthy controls were collected at regular screening colonoscopies at the University Hospital Zurich. Patients had to be older than 18. Endoscopic disease activity was assessed using the Mayo Clinic endoscopy subscore for UC and the CD endoscopic index of severity (CDEIS) for CD, respectively. (24-26) A score of ≥1 (Mayo Score) and ≥3 (CDEIS) was considered as active disease. (25, 27) Patients were excluded for: i) biological treatment with anti-TNF within the last 8 weeks, ii) ongoing treatment for inflammatory skin disorder (topical or systemic), iii) concomitant autoimmune disorders such as systemic lupus erythematodes or vasculitis, and iv) concomitant skin disorder such as eczema, atopic dermatitis or paradoxical anti-TNF induced skin lesions. Clinical data were retrieved from paper-based and electronic patient

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records. The following data were collected: patient demographics (gender, age), disease history, previous and current medications and comorbidities. For this purpose, a standardized spreadsheet was used (Supplementary Table 1).

Tissue sample collection

For intestinal samples, biopsies were taken from the terminal ileum (TI), cecum, and colon using a needle forceps. For skin samples, biopsies were taken from the affected lesion using a 4-6 mm surgical punch that collected epidermis, dermis and the upper subcutis. For healthy control skin, fresh, non-affected and non-inflamed skin of unrelated surgical excisions was used. Biopsies were formalin fixed-paraffin embedded and stored at -80°C for further use.

Tissue microarray and immunostaining

Tissue microarrays (TMA) were constructed using formalin fixed paraffin-embedded tissue blocks, which had been punched out from ileal, cecal, colonic, sigmoid or skin tissue by a tissue cylinder (0.6mm in diameter), as it had been previously described. (28, 29) Immunohistochemical staining was performed using the automated system BOND RX (Leica Biosystems). TMA sections were deparaffinized and rehydrated in dewax solution (Leica Biosystems). Immunohistochemistry was carried out for the TNF-NFκB (TNFα, NFκB) and JAK-STAT pathway (STAT1, STAT3) as well as the MAdCAM-integrin interaction (MAdCAM1), which all represent targets of IBD treatment modalities. We further investigated markers for B lymphocytes (CD20), macrophages (CD68), Th1-cytokine mediated disease (IFNγ), apoptosis (caspase 3 and 9), and inflammation-induced antiapoptotic factors (Hsp-27/70). The following primary antibodies were used: TNFα (Abcam, ab6671, dilution 1:50), NFκB p65 (Abcam, ab32360, dilution 1:100), MAdCAM1 (clone CA102.2CI, Affimetrix BMS170, dilution 1:1000), STAT1 (R&D Systems, MAB1490, dilution 1:50), STAT3 (Abcam, ab50761-100, dilution 1:100), CD20 (Ventana Roche, prediluted), CD68 (Ventana Roche, prediluted), caspase 3 (Abcam, ab4051, dilution 1:200), caspase 9 (Invitrogen, PA5-17913, dilution 1:200), IFNγ (BD Biosciences, 560371, dilution 1:100), Hsp-27 (Leica Biosystems, NCL-HSP27, dilution 1:40), and Hsp-70 (Enzo Life Sciences, dilution 1:100). Antibody was detected with the Bond Polymer Refine Detection kit (Leica Biosystems), using DAB (3-3'-Diaminobenzidine) as chromogen and following the manufacturer's instructions. A semiquantitative scoring system ranging from 0 to 100% (5% intervals) was used for evaluation of immunostaining. (29, 30) Scoring was performed by two reference pathologists in the field of tissue microarrays (AL, HD). TMA review was blinded. All samples and

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controls were run in duplicate. Failure of analysis occurred in 15.1% (796/5040 spots) of all gastrointestinal samples and in 9.4% (72/765 spots) of skin samples. Reasons for such failure were missing samples (empty spots on microarray) or TMA technology.

Statistical analysis

For all statistical analyses, IBM software SPSS (version 22.0.0, 2013 SPSS Science, Inc., Chicago, IL) was used. Each biopsy sample was analyzed individually. Metric data are presented as means and standard deviation (SD). Categorical data are depicted as percentage of the group total. For comparisons between continuous variables, two-sample t-test and Mann-Whitney-U test were used depending on whether data were normally distributed or not. A two-sided p-value of <0.05 was regarded as statistically significant.

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RESULTS

Tissue samples

A total of 304 intestinal and skin tissue samples were collected. 240 intestinal biopsies were taken from a total of 106 patients. These samples showed active endoscopic IBD in 108 cases and endoscopic inactivity in 77 cases. 55 intestinal biopsies were taken from 29 healthy controls. 64 skin samples were collected from 64 patients, 53 patients had an inflammatory skin disorder (18 EN, 13 PG, 22 psoriasis), while 11 patients (controls) had normal skin. 2 of the 53 patients with an inflammatory skin disorder (1 EN and 1 PG) had corresponding intestinal biopsies showing active ileocecal CD (for EN) and active UC pancolitis (for PG). In detail, the following samples were analyzed: 74 from TI (19 HC, 19 CD inflamed, 13 CD not inflamed, 23 UC not inflamed), 79 from the cecum (21 HC, 17 CD inflamed, 13 CD not inflamed, 28 UC not inflamed), 57 from the sigmoid colon (15 HC, 42 UC inflamed), 30 from the colon (30 UC inflamed), and 64 from the skin (11 HC, 18 EN, 13 PG, 22 Psoriasis).

Patient demographics

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156 (51.3%) samples were taken from male patients, mean age was 43.7 years (SD 9.7). Patients with PG were significantly older, and there were significantly less males in the EN group, the other groups were comparable regarding gender and age. Of the 185 IBD intestinal samples, 36 showed endoscopically active CD, 26 non-active CD, 72 endoscopically active UC and 51 showed non-active UC. Of the 72 samples with endoscopic UC activity, 31 UC samples were collected from patients with pancolitis, while 41 from patients with left sided UC. All of the 36 samples with active CD were taken from ileal (n=19) or cecal (n=17) disease, while the samples for inactive CD were collected from non-inflamed TI (n=13) or non-affected cecum (n=13). Two IBD patients reported a previous anti-TNF treatment, which was however stopped more than 8 weeks before biopsy. At the time of skin biopsy, none of the patients with skin disorders were treated with systemic or topical anti-inflammatory drugs. None of them had a history of a previous use of biological treatment. None of the patients with psoriasis had underlying IBD. Table 1 summarizes demographic data and prior medication of all patients and the respective subgroups.

Expression of the TNFα/NFκB pathway in intestinal disease and cutaneous manifestations

Subepithelial TNFα expression was significantly higher in active CD compared to controls (36.2 vs. 12.1, p<0.001), but was not elevated in inactive CD (20.9 vs. 12.1, n.s.). Difference between active and inactive CD was also significant (p=0.019). No difference was seen regarding TNFα expression in UC patients (regardless of disease activity) vs. controls (17.9 and 13.1 vs. 12.1, n.s.). TNFα expression was significantly higher in CD than in UC (36.2 vs. 17.9, p=0.001). However, NFκB, a downstream signal of TNF was upregulated in both active UC (34.5) and active CD (43.2) vs. controls (21.8, p=0.017, p<0.001) with no significant differences between the two diseases. In inactive disease, NFκB was not significantly elevated neither in CD nor in UC. Similarly to what was seen in intestinal samples, subepithelial TNFα was overexpressed in all inflammatory skin disorders (EN 25.6, PG 37.7, psoriasis 14.7) compared to controls (3.2, p<0.001, p=0.001, and p=0.001). PG demonstrated even higher TNFα expression levels than psoriasis (37.7 vs. 14.7, p=0.022), while for EN vs. psoriasis at least a clear trend was seen (25.6 vs. 14.7, p=0.052). Similar results were observed for NFκB expression with however no difference between PG and psoriasis, while upregulation of NFκB was significant in EN vs. psoriasis (p=0.002). NFkB expression was 41.8 in EN, 25.0 in PG, 14.3 in psoriasis and 5.9 in healthy controls. For details regarding TNFα and NFκB expression see Figures 1 and 2.

Expression of MAdCAM1

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MAdCAM1 was upregulated in inflamed intestinal tissue. Compared to healthy controls, active UC showed a significant MadCAM1 overexpression (57.5 vs. 35.4, p=0.003). MadCAM1 was also upregulated in active UC when compared to inactive UC (57.5 vs. 39.3, p=0.027). Expression from intestinal samples with active CD did not show significant differences when compared to healthy controls (30.8 vs. 35.4). In contrast to the upregulation of MAdCAM1 in UC patients, no overexpression compared to healthy controls was seen for EN, PG, and psoriasis. In fact, expression levels of MAdCAM1 were below the threshold of 10% in all samples with inflammatory skin disorders. Figure 3 shows representative tissue samples from the intestine (IBD and healthy controls, Fig. 3a) and inflammatory skin diseases (Fig. 3b) stained for TNFα and MAdCAM1. Representative tissue samples for inactive intestinal disease stained for TNFα and MAdCAM1 are depicted in Supplemental Figure 1. For expression levels of intestinal samples see Supplemental Figure 2.

Expression of STAT proteins in intestinal disease and cutaneous manifestations

STAT3, which is involved in the JAK-STAT pathway and which can be blocked by JAK inhibitors such as tofacitinib, was elevated in both active and inactive CD (73.4 and 81.5 vs. 60.5, p=0.028 and p=0.002). Same results were seen for active and inactive UC (79.2 and 72.7 vs. 60.5, p<0.001 and p=0.012). There was no difference between active and inactive disease nor between UC and CD (n.s.). Of note, same significant upregulation of STAT3 was observed in EN (84.7) and PG (60.5) compared to controls (22.3) and psoriasis (26.8). STAT3 was even higher expressed in EN compared to PG (p=0.033). For details see Figure 4. Although there was also some significant overexpression of STAT1 in EN, absolute numbers (EN 17.1, PG 1.4, psoriasis 2.9, controls 0.0)) were considerable lower than those seen for STAT3.

Expression of other proteins

We assessed the expression of several other proteins involved in inflammatory processes and cell injury. Caspase 3 upregulation significantly distinguished PG and EN from healthy controls and psoriasis: Caspase 3 was higher in both EN (20.0) and PG (17.7) vs. in psoriasis (2.8, p<0.001 and p=0.002) and in controls (0.9, p<0.001, and p<0.001). Caspase 9 was overexpressed in EN (13.1), PG (10.9), and psoriasis (6.0) compared to controls (2.3), although upregulation in PG was not significant due to a high variation. Nonetheless, these results highlight caspases as potential targets in EIM management. While no upregulation of IFNγ in intestinal disease nor in skin lesions was seen, there was a significant overexpression of CD68 in inflammatory skin disorders (EN 54.7, PG 32.3, psoriasis 10.2 vs. controls 3.9, p<0.001, p<0.001, and p=0.003, respectively). Upregulation was significantly higher in EN compared to PG (p=0.042) and psoriasis (p<0.001). Difference between PG and psoriasis

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was also significant, highlighting the possible pathogenic role of CD68 and therefore macrophages in PG and EN pathophysiology, but not in psoriasis. However, absence of CD68 overexpression in intestinal IBD, as well as low absolute numbers of CD20 (controls 0.0, EN 2.2, PG 2.1, psoriasis 1.4) and Hsp-27 expression (controls 5.9, EN 11.8, PG 6.8 and psoriasis 4.5) do not allow drawing clear conclusions. Hsp-70 did not show any overexpression in the skin samples (controls 8.2, EN 9.4, PG 8.2 and psoriasis 5.6). Expression levels of Caspase 3, Caspase 9, Hsp-27, Hsp-70, CD20, CD68 and IFNγ are depicted in Supplemental Figure 3.

DISCUSSION

This single-center observational study with assessment of protein expression patterns in IBD, cutaneous EIM and psoriasis compared to healthy controls shows similar upregulation of TNFα and STAT3 in both intestinal and cutaneous disease, but absence of MAdCAM1 overexpression in inflammatory skin disorders. TNFα and STAT3 are both considerably upregulated in EN and PG with even higher expression levels compared to psoriasis. Protein expression patterns of EN and PG show many similarities with only few differences revealing important therapeutic implications.

The TNFα-NFκB axis is overexpressed in both intestinal disease as well as in inflammatory skin disorders with an even more pronounced upregulation in PG and EN compared to psoriasis. TNFα was recognized as an important inflammatory parameter in different autoimmune diseases including IBD and psoriasis many years ago. Anti-TNF take advantage of this canonical inflammatory pathway. They have been successfully introduced in the last decade and have changed IBD management dramatically. Their potential beneficial role in EIM management had been first proposed based on small case series and case reports. (31) Very recently, Peyrin-Biroulet and colleagues nicely summarized their efficacy in a meta-analysis demonstrating high response rates for PG (69-100%) and EN (prevalence reduction from 2.4 to 0.4%). Complete remission rates for PG were however lower (25-100%). (18, 19) PG actually is the only cutaneous IBD manifestation where a randomized controlled trial is available for showing clinical improvement in 69% and a complete remission in 25% of the patients after an induction treatment with infliximab. (32) Further data from the Swiss IBD cohort study supports this data demonstrating improvement rates of 60% for PG and 80% for EN. (33) This has led to the current understanding of a TNF-dependent pathogenesis in cutaneous EIM. While in PG samples indeed TNFα upregulation has been reported previously, no such data is

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available for EN. Our data of overexpression in both PG and EN, and even higher levels compared to psoriasis, are compatible with the known efficacy of anti-TNF efficacy in cutaneous EIM. (16) Although TNFα-NFκB axis was upregulated in both CD and UC with a particular overexpression of intracellular NFκB, TNFα was actually only significantly increased in intestinal samples from active CD, but not in UC. There are three possible explanations: 1) The finding that TNFα levels in the lamina propria are indeed higher in CD than UC has been already described more than two decades ago. (34) 2) For detection of the small difference between healthy controls and UC our study was probably underpowered. 3) Other factors such as bacterial products (e.g. lipopolysaccharides, LPS) can lead to NFκB upregulation independent from TNFα, so (over)expression of the two proteins do not necessarily go in parallel.

The anti-integrin vedolizumab has been introduced into clinical practice very recently. Data from randomized controlled trials demonstrated vedolizumab`s efficacy in both CD and UC. (2, 3) Vedolizumab`s role in EIM management has not yet been defined. Its gut-selective mechanism advocates against, while some preliminary data presented at recent congresses advocate for an at least partial efficacy in the treatment of EIM. (35) In consistence with the literature, we herein report on isolated MAdCAM1 expression in the inflamed intestine, but not in inflammatory skin disorders, which makes a direct effect on cutaneous EIM extremely unlikely. If anyhow, vedolizumab might have a role in EIM management by treating intestinal disease activity in EIM that parallels IBD.

The third biologic, which is currently reviewed by the US FDA for treatment in UC patients, is the JAK inhibitor tofacitinib, which has been successfully used in other diseases such as rheumatoid arthritis and psoriasis. (20, 21) Tofacitinib inhibits the JAK-STAT pathway, which incites pro-inflammatory signals. Biopsies from active intestinal disease and inflammatory skin disorders demonstrated similar results regarding STAT3 overexpression in our patient cohort. Difference between healthy controls and inflamed tissue was even more prominent in skin biopsies. Of note, STAT3 was significantly more upregulated in PG/EN than in psoriasis shedding light on the potential benefit from JAK inhibition in those patients. Clinical data is not available so far, but our findings make a therapeutic response to JAK inhibitors very likely. Given tofacitinib`s current blackbox warning for serious infections, possible systemic side-effects from pan-JAK inhibitors should be kept in mind, which however occur less frequent with more specific JAK inhibitors such as filgotinib.

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With the microarray approach, several other interesting targets for EIM management have been identified by screening markers for B-lymphocytes (CD20), macrophages (CD68), apoptosis (caspase 3 and 9) and inflammation-induced anti-apoptotic factors (Hsp-27/70). Both caspase 3 and CD68 were overexpressed in EN and PG compared to healthy controls and psoriasis highlighting the overlap between cutaneous EIM with a protein expression profile distinct from other inflammatory skin diseases. Anti-caspases are currently tested in liver diseases (emricasan in non-alcoholic steatohepatitis, NCT02686762), while no data is available so far for EIM or IBD.

Our study has several strengths, but also some limitations. To the best of our knowledge, it is so far the only study investigating protein expression patterns in IBD and cutaneous EIM in IBD comparing them to healthy controls and psoriasis. Microarray is a widely used and reliable tool to study expression of various proteins. Variation due to TMA technology has been limited using only duplicate samples. None of the patients with inflammatory skin disorders were treated with neither topical nor systemic anti-inflammatory drugs, which makes the TMA results more reliable. In addition, previous anti-TNF treatment was reported in two IBD patients only, and treatment was stopped more than 8 weeks before study evaluation, making interference with biopsy results less likely. All TMA were analyzed by experts in TMA evaluation and involvement of only two pathologists has limited inter-observer variability. We were able to include 13 patients with PG despite its rarity (prevalence 2% of Swiss IBD population, incidence 0.63/100`000) due to the close collaboration with our Department of Dermatology and a study enrolment over several years. (15, 36) A limitation of the TMA technology is that tissue sample heterogeneity is not completely taken into account. The study was cross-sectional without any follow-up, therefore changes in expression levels in response to any biological treatment could not be assessed. The study relied on protein expression levels only and did not include analysis of mRNA levels. A further drawback of this study is that we only identified MAdCAM1 overexpression in active UC, but not active CD samples. This might be due to 1) the low number of samples from active CD (n=36) compared to the relatively higher number of active UC (n=72) and 2) the inclusion of non-severe CD cases given the very low cut-off of CDEIS ≥3. (37)

While upregulation of TNFα in cutaneous EIM compared to healthy controls is compatible with the known efficacy of anti-TNF in EIM management, the lack of MAdCAM1 overexpression in the human skin, even in the context of inflammation, questions the role of vedolizumab in EIM treatment. It`s only effect might be indirect through inducing and maintaining intestinal disease remission in those EIM paralleling IBD activity. Data on upregulated STAT give a rationale for JAK-inhibitors in both EN

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and PG management, particularly in the current context of missing clinical studies. Data from randomized controlled trials evaluating the effect of anti-integrins and JAK-inhibitors on EIM are however urgently needed.

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AUTHORSHIP STATEMENTS

SRV and TG take responsibility for the integrity of the work as a whole, from inception to published article. Author contributions: Study conception (AL, IZ, SRV and TG), data collection (AMS, GR, LB, MBP, MS, SRV, TG), tissue microarray immunohistochemistry and analysis (AL, AS, HD, IZ, JAG, LT), data analysis (AMS, SRV and TG), drafting of manuscript (SRV and TG), critical review of manuscript (AL, AMS, AN, AS, GR, IZ, JAG, LB, LT, MBP, MS). All authors approved the final version of the manuscript.

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TABLES:

	All samples n=304	Intestinal samples from IBD N=185	Skin samples from EN N=18	Skin samples from PG N=13	Skin samples from Psoriasis N=22	Skin and intestinal samples from Controls N=66
Age						
- mean in years (SD)	43.7 (9.7)	41.9 (14.5)	43.8 (20.7)	61.0 (17.4)	48.7 (16.3)	43.1 (10.6)
Gender						
males	156 (51.3%)	101 (54.6%)	5 (27.8%)	6 (46.2%)	12 (54.5%)	32 (48.5)
Concurrent medications						
- none	164 (53.9%)	50 (27.0%)	18 (100%)	13 (100%)	22 (100%)	61 (92.4%)
- 5-ASA	107 (35.2%)	106 (57.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.5%)
- topical steroids	15 (4.9%)	15 (8.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- syst. steroids	21 (6.9%)	21 (11.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- antibiotics	10 (3.3%)	8 (4.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (3.0%)
- AZA	9 (3.0%)	9 (4.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- Antihistamines	4 (1.3%)	4 (2.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- Biologics	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- NSAR	3 (1.0%)	1 (0.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (3.0%)
Previous biological treatment						
- anti-TNF	2 (0.7%)*	2 (1.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- vedolizumab	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
- JAK inhibitor	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Surgical history	2^{+} (0.7%)	2^{+} (1.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

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Table 1: demographic data and prior medication. * Treatment was stopped 8 weeks before biopsies, [+] ileocecal resection (1), partial colectomy (1). 5-ASA, aminosalicylates; AZA, azathioprine; EN, erythema nodosum; IBD, inflammatory bowel disease; NSAR, non-steroidal antirheumatic agent; PG, pyoderma gangrenosum.

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FIGURE LEGENDS:

Figure 1: Mean TNF expression in intestinal and skin tissue. Standard error of the mean bars are shown. P-value calculated by Mann-Whitney-U test.

Figure 2: Mean NFkB expression in intestinal and skin tissue. Standard error of the mean bars are shown. P-value calculated by Mann-Whitney-U test.

Figure 3 Immunohistochemical analysis of intestinal and skin tissue TMA for TNF and MAdCAM1 protein expression.

a) intestinal tissue; A-C: MAdCAM1 immunostaining and D-F: TNF immunostaining; A/D) normal colon, B/E active CD, C/F active UC.

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b) skin tissue; A-D: MAdCAM1 immunostaining and E-H: TNF immunostaining; A/E) normal skin, B/F erythema nodosum, C/G pyoderma gangrenosum, D/H psoriasis.

Figure 4: Mean STAT3 expression in intestinal and skin tissue. Standard error of the mean bars are shown. P-value calculated by Mann-Whitney-U test.

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Figure 1.

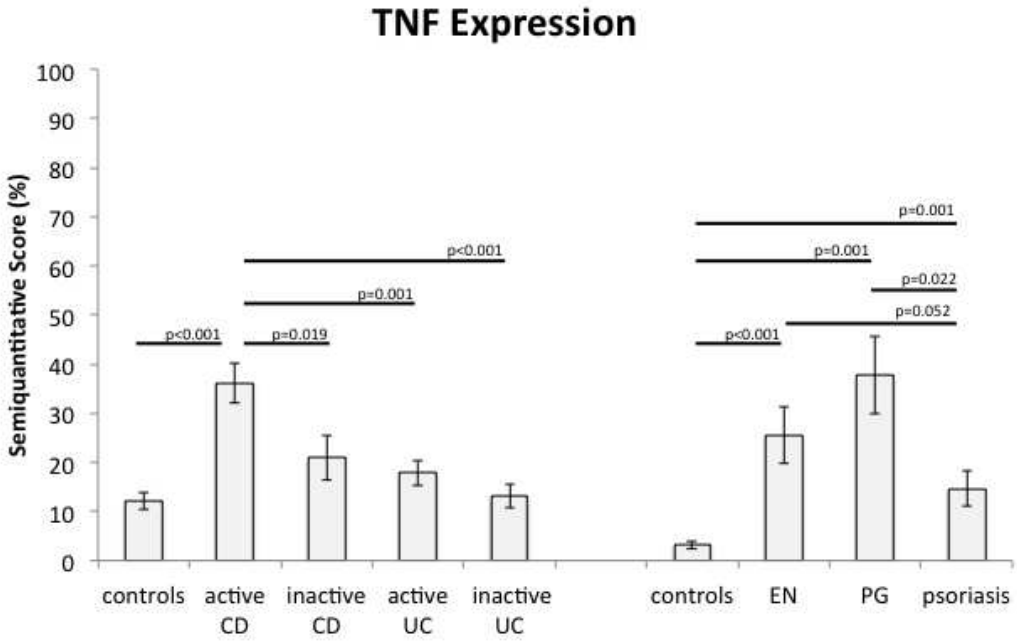

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Figure 2.

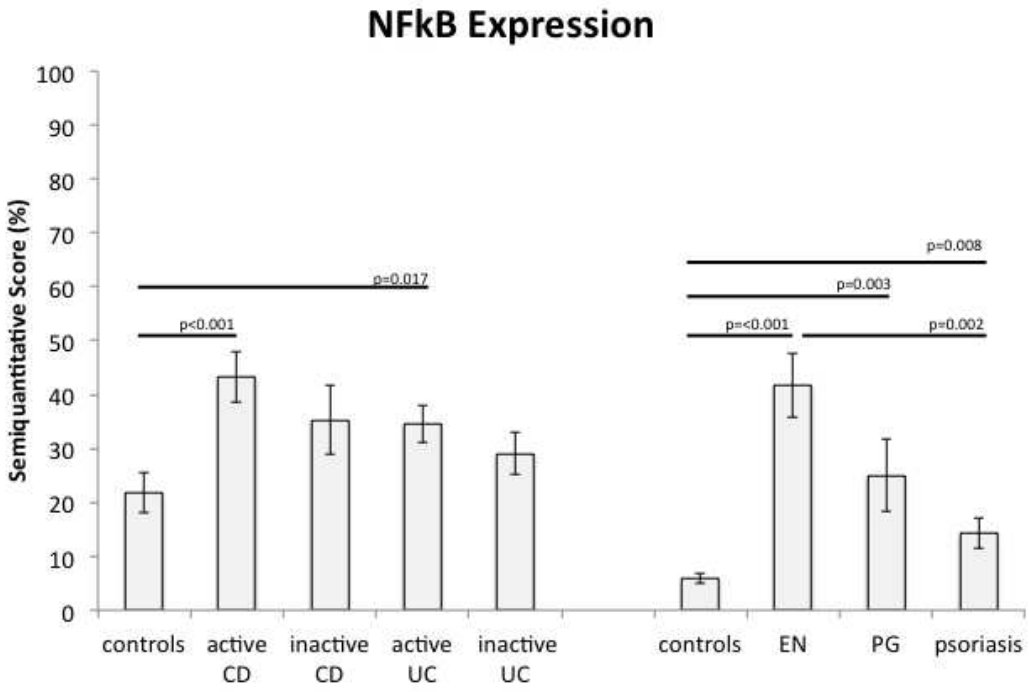

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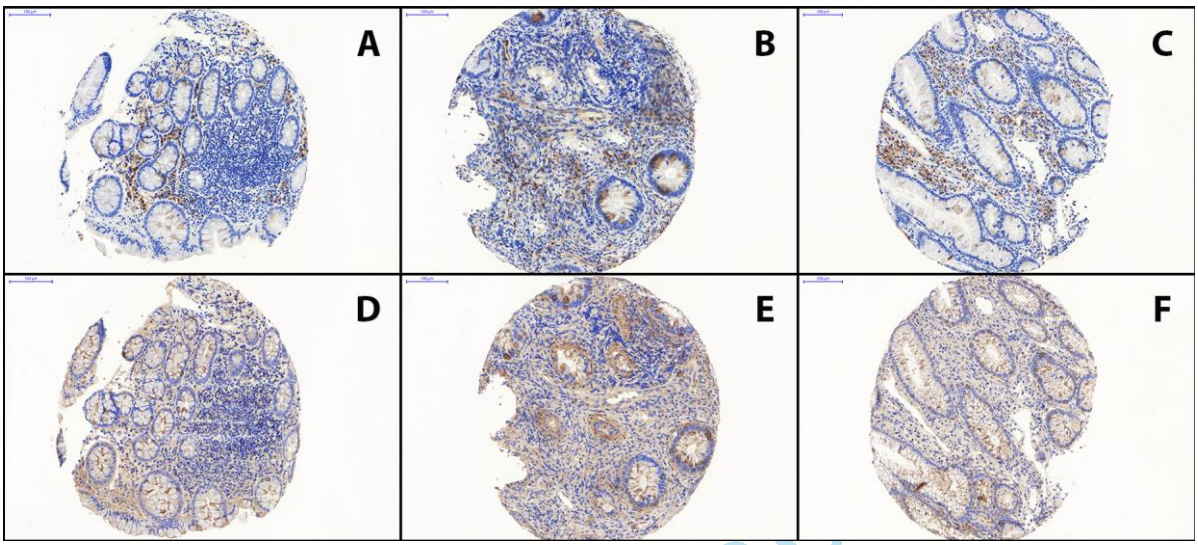
Figure 3.

a)



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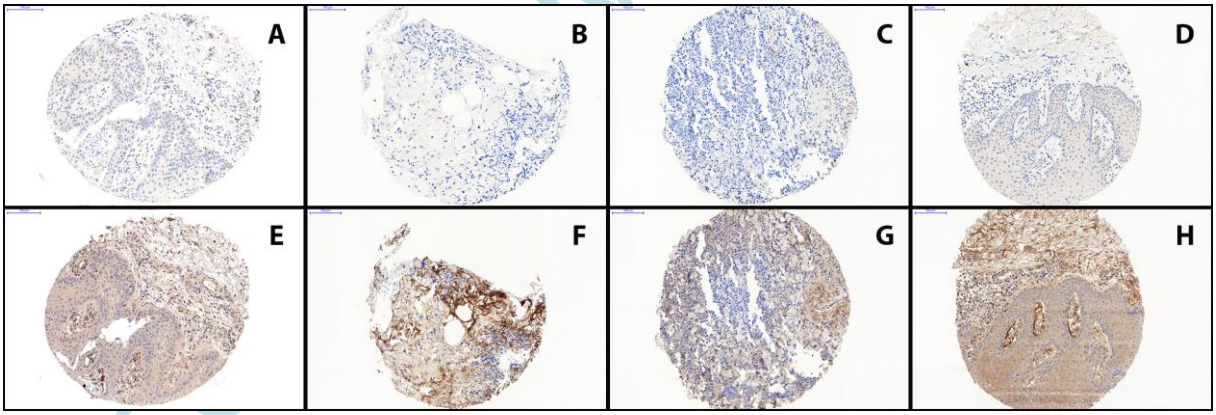

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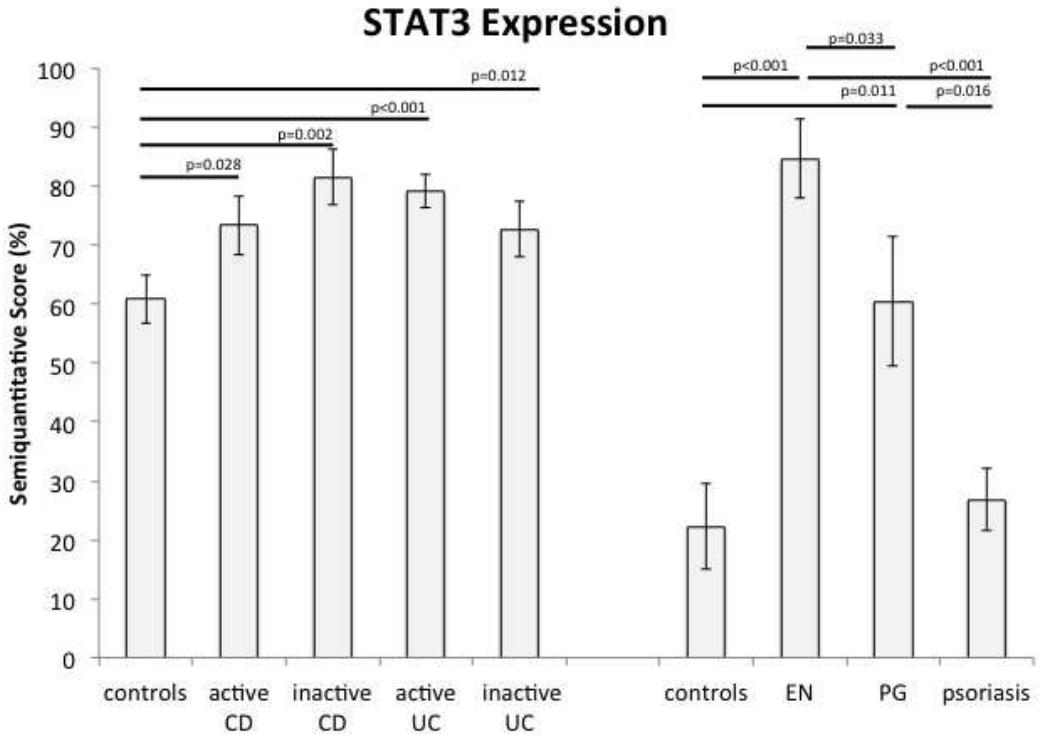
Figure 4.



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