

Serological Assessment of the Quality of Wound Healing Processes in Crohn's Disease

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ABSTRACT

Background & Aims: Crohn's disease (CD) is a chronic inflammatory condition characterized by continuous mucosal damage and ongoing wound healing of the intestines. The fibrinolytic system is involved in early parts of the wound healing process. Fibrin is a key mediator of primary blood clot formation and is formed by cross-linking of fibrinogen. To gain insights into the dynamics of wound healing in CD patients we investigated the conversion of fibrinogen into fibrin by the pro-peptide FPA, the amount of factor XIII cross-linked fibrin and total fibrin clot.

Methods: Serum samples of 35 CD patients, 15 non-inflammatory bowel disease (non-IBD) patients and 39 age-matched healthy controls were analyzed for three novel neo-epitope markers: D-fragment and D-dimer, reflecting the degradation of total fibrin clot and factor XIII cross-linked fibrin, as well as FPA, reflecting synthesis of fibrin.

Results: Crohn's disease patients had a significantly lower D-dimer level ($p=0.0001$) compared to healthy controls. Crohn's disease and non-IBD patients had a significantly higher level of FPA ($p<0.0001$) and D-fragment/D-dimer ratio ($p<0.0001$ and $p=0.02$). FPA, D-dimer and D-fragment/D-dimer ratio could distinguish CD patients from healthy controls with area under the curve of 0.92 (95% CI 0.83-0.97), 0.78 (95% CI 0.67-0.87) and 0.85 (95% CI 0.75-0.93), respectively.

Conclusion: Wound healing parameters were clearly changed in CD patients. FPA levels were higher in CD patients as compared to healthy controls, indicating more ongoing wound healing. D-dimer levels were lower in CD patients than in healthy controls, indicating impaired wound healing due to poor quality of factor XIII cross-linked fibrin and clot resolution.

Key words: fibrinogen – fibrin – Crohn's disease – D-dimer – fibrinopeptide A.

Abbreviations: CD: Crohn's disease; FXIII: factor XIII; FgDP: fibrinogen degradation products; FnDP: fibrin degradation products; FPA: fibrinopeptide A; IBD: inflammatory bowel disease; PAI-1: plasminogen activator inhibitor-1; tPA: tissue plasminogen activator.

INTRODUCTION

Crohn's disease (CD) is one of the main inflammatory bowel diseases (IBD) [1]. It is characterized by chronic inflammation of the intestines with impaired wound healing due to continued tissue destruction and repair of the endothelium and epithelium [2]. Dysfunction and dysregulation of the wound healing response may lead to a sustained intestinal inflammation accompanied by poor quality of

wound healing, leading to complications such as ulcers, fibrosis and fistula formation in the face of reversal.

Non-healing wounds have traditionally been defined as those that fail to progress through an orderly sequence of repair in a timely fashion [3]. During early wound healing, platelets are activated and recruited at the wound site where clot formation and fibrinolysis occur. Fibrinogen is cross-linked by transglutaminase factor XIIIa (FXIIIa) and converts into fibrin to form fibrin-rich clots, while fibrinopeptide A (FPA) is cleaved from fibrinogen. Subsequently, the fibrinolytic system, which includes a series of enzymes and inhibitors, degrades fibrin in order to dissolve the clot, while D-dimer is cleaved from fibrin [4].

Any imbalance in the fibrinolysis pathways may affect the quality of wound healing and lead to hypo- or hyperfibrinolysis,

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both of which have been described in IBD [4]. The activator of fibrinolysis-tissue plasminogen activator (tPA) has been reported to be decreased and the inhibitor of fibrinolysis-plasminogen activator inhibitor-1 (PAI-1) is increased in IBD patients, clearly suggesting an imbalance [5, 6] of hypofibrinolysis in IBD. In contrast, FXIIIa has been reported to be decreased in IBD patients while FXIIIb is not significantly changed, suggesting that decreased FXIIIa could be consequent to the rapid consumption during clot formation [7, 8]. Moreover, the end products of fibrinolysis, plasma level of D-dimer and fibrinogen degradation products (FgDP) are increased in IBD, which indicates hyperfibrinolysis [7–11]. In direct alignment, the fibrin clot structure has been reported to be altered in IBD patients, with a more dense fibrin network resistant to resolution by plasmin [12]. Overall, stable clot formation is essential for proper wound healing. The imbalance in the fibrinolysis pathways leads to inappropriate wound healing and may contribute to the development and progression of CD [4].

Although commercial assays are available to measure fibrinogen and fibrin degradation products, they are targeting the conformation change or unidentified epitopes. Therefore, we developed specific assays targeting the neo-epitope from fibrin(ogen) cleaved by plasmin. A monoclonal antibody recognizing the plasmin cleavage site of fibrin(ogen) β -chain was applied in both a competitive and a sandwich ELISA. Total clot and cross-linked fibrin clot are measured by two different assays. By using the competitive ELISA (named as “D-fragment”), both FgDP and fibrin degradation products (FnDP) can be measured. By using the sandwich ELISA (named as “D-dimer”), only D-dimer, which is cleaved from FXIII cross-linked fibrin by plasmin can be measured. We investigated the processes of wound healing in CD patients by studying the turnover of fibrinogen and cross-linked fibrin. In addition, the formation marker of fibrin, FPA, was determined, to gain information about the quality of the wound healing processes in CD.

MATERIALS AND METHODS

Crohn's disease clinical study

The clinical study has been described elsewhere [13]. Briefly, the study was a prospective blinded multicenter study (ClinicalTrials.gov NCT01019460). In this study, 72 patients were diagnosed with CD. Location and disease behavior were assessed by the Montreal classification. Thirty-two patients were diagnosed with non-IBD; they had a standardized work-up including ileocolonoscopy, capsule endoscopy, magnetic resonance enterography or computer tomography enterography. Serum samples were stored at $\leq -20^{\circ}\text{C}$ until further measurements. As fibrinogen has different expression levels and synthesis rate in adolescents as compared to adults [14], only adults aged above 25 were included in the biomarker measurement. Therefore, serum from 35 CD patients and 15 non-IBD patients was measured. Patients' demographics are summarized in Table I. The non-IBD group, mean age 42.5 years, included patients with irritable bowel syndrome (n=10), functional diarrhoea (n=3), chronic appendicitis (n=1) and

Table I. Patients' demographics

	CD (n=35)	Non-IBD (n=15)
Gender		
Female, (%)	23 (66)	12 (80)
Male, (%)	12 (34)	3 (20)
Age Mean; (range)	44.0 years (26-72)	42.5 years (26-75)
Use of corticosteroids		
Yes (%)	10 (29)	0 (0)
No (%)	25 (71)	15 (100)
Disease activity (CDAI score)		
Active disease (>150) (%)	16 (46)	N/A
Remission (<150) (%)	19 (54)	N/A
Montreal classification Behaviour		
B1	18	N/A
B2	14	N/A
B3	3	N/A
C-reactive protein		
>5mg/L (%)	12 (34)	8 (53)

coeliac disease (n=1). Serum samples from 39 age-matched healthy controls (Valley Biomedical) were included as controls.

Three biomarkers were measured in the serum: fibrinopeptide A (FPA, in-house ELISA assay), D-fragment competitive assay and D-dimer sandwich assay.

Antibody development for D-fragment and D-dimer assays

All peptides were purchased from the American Peptide Company. We used the 10 amino acids from newly exposed N-terminus of plasmin cleaved fibrin(ogen) β chain, 164'DNENVVNEYS'173 as the immunogenic peptide to generate specific neo-epitope monoclonal antibodies. 6-7-week-old Balb/C mice were immunized subcutaneously with 200 μL Sigma adjuvant (S6322, Sigma) emulsified antigen with 100 μg of the immunogenic peptide. Consecutive immunizations were performed at 2-week intervals until stable sera titer levels were reached. The mouse with best native reactivity was selected for fusion.

The fusion procedure has been described elsewhere [15]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The fusion cells were raised in 96-well plates and incubated in the CO₂-incubator. Here, standard limited dilution was used to promote monoclonal growth. Cell line 3A9 specific to the selection peptide (DNENVVNEYS) and without cross-reactivity to elongated peptide (KDENVVNEYS) or truncated peptide (NENVVNEYS) was selected and sub-cloned. At last, the antibody was purified by an IgG column (GE Healthcare).

In vitro cleavage of fibrinogen and fibrin

Five mg of fibrinogen (F3879, Sigma)/12.5mg of fibrin both from human plasma (F5386, Sigma) were dissolved/suspended in 0.5 mL 50 mM Tris-HCl buffer, pH 7.6. 0.25 nKat or 1.07

nKat of plasmin (10602361001, Sigma) was added to fibrinogen solution or fibrin suspension respectively. The mixture was incubated at 37°C for 4 hours. The reaction was terminated by adding soybean trypsin inhibitor (T9003, Sigma). The supernatant was stored at -20°C after centrifuge.

D-fragment competitive ELISA and D-dimer sandwich ELISA protocol

ELISA-plates were Streptavidin-coated from Roche (cat.: 11940279). All ELISA plates were analyzed with ELISA reader from Molecular Devices, SpectraMax M. For D-fragment competitive ELISA, 96-well plate was coated with biotinylated peptide DNENVVNEYSK-Biotin for 30 minutes at 20°C. Twenty μ L of standard peptide or samples were added to appropriate wells, followed by 100 μ L of monoclonal antibody 3A9, and incubated 3 hours at 4°C. After plate wash, 100 μ L of Horseradish Peroxidase (HRP) labeled rabbit anti-mouse secondary antibody (Jackson ImmunoResearch) was added and incubated at 20°C for 1 hour. For D-dimer sandwich ELISA, the plate was coated with biotinylated antibody 3A9 for 30 minutes at 20°C. 20 μ L of standard dilution or samples followed by 80 μ L buffer were added to appropriate wells and incubated 20 hours at 4°C. After plate wash, 100 μ L of HRP labeled antibody 3A9 was added and incubated at 20°C for 1 hour. Both competitive and sandwich ELISA have the same colorimetric determination step. 100 μ L tetramethylbenzidine (TMB) (Kem-En-Tec cat.438OH) was added and the plate was incubated 15 minutes at 20°C in dark. The TMB reaction was stopped by adding 100 μ L of stopping solution (1% H₂SO₄) and measured at 450 nm with 650 nm as a reference. All the above incubation steps included shaking at 300 rpm. After each incubation step, the plate was washed five times.

Technical evaluation for D-fragment competitive assay and D-dimer sandwich assay

The lower limit of detection (LLOD) was determined from 21 zero samples (buffer) and calculated as the mean and 3x

standard deviation (SD). Upper limit of detection (ULOD) was determined as the mean and 3x SD of 10 measurements of Standard A. The intra-assay and inter-assay variation were the mean variations of seven samples run ten independent times in duplicate. Dilution recovery was determined in three serum and plasma samples (Valley Biomedical) and was calculated as a percentage of recovery of diluted samples from undiluted samples. Spike recovery was calculated by comparing different concentrations of peptide solution in buffer and in human serum/plasma samples. Interference was determined in 90 ng/ml Biotin, 5 mg/ml hemoglobin, 5 mg/ml intralipid and a high level of human anti-mouse antibody (HAMA) samples. Sample stability was determined in samples freeze-thaw up to four cycles and calculated the recovery from the non-freeze-thaw samples.

Statistics

Statistical analysis was performed using MedCalc version 14 and GraphPad Prism version 7. The biomarker levels were presented as mean values and standard error of the mean (SEM). The differences of D-fragment and D-dimer among non-IBD, CD and healthy controls were determined by Kruskal-Wallis one-way ANOVA test, Dunn's multiple comparisons test. The diagnostic power of biomarkers was investigated by the area under the receiver-operating characteristics (ROC) curve (AUC) with 95% confidence interval (CI). Sensitivity and specificity were determined for appropriate cut-off values based on the ROC curves. The correlations between biomarkers and disease parameters were determined by nonparametric Spearman correlation. Significance threshold was set at $p < 0.05$.

RESULTS

Characterization of D-fragment competitive assay and D-dimer sandwich assay

When applied in a competitive ELISA (Fig. 1A), the chosen antibody 3A9 specifically recognized selection peptide

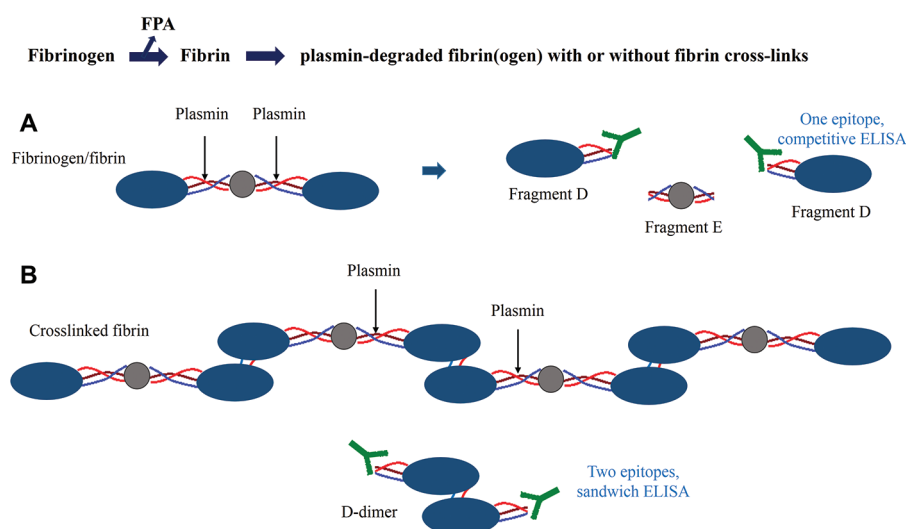


Fig. 1. Schematic overview of antibody recognition of fibrinogen/fibrin. Fibrinogen is a dimer composed of two sets of α , β and γ chains which binding together by disulfide bonds. Transglutaminase factor FXIIIa (FXIIIa) cross-link γ - γ , α - α or α - γ fibrin chains creating multimeric forms of crosslinked fibrin. Plasmin degrades fibrin(ogen) releasing the D-fragments and D-dimers. (A) Competitive ELISA, D-fragment. (B) Sandwich ELISA, D-dimer.

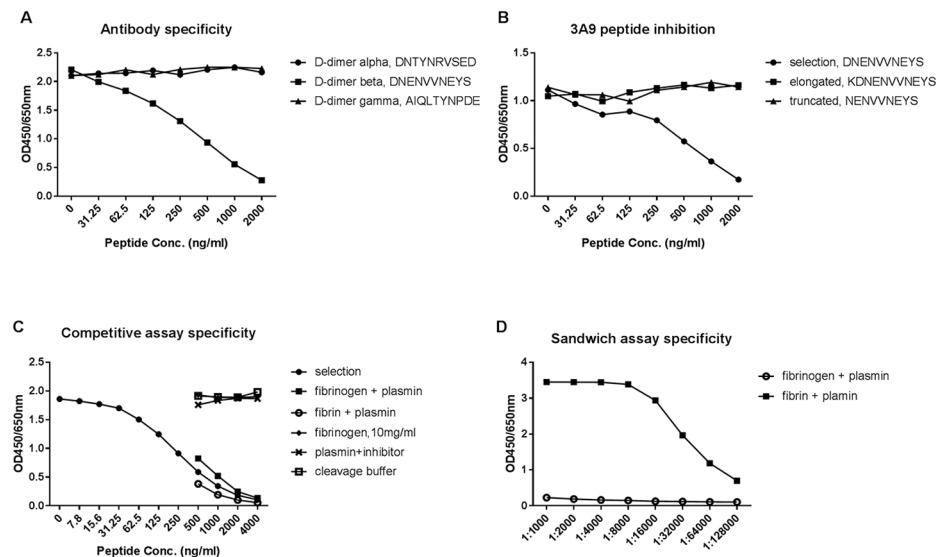


Fig. 2. Cell line 3A9 reaction with fibrinogen/fibrin cleavage products produced by plasmin. (A) Antibody reactivity towards N-terminus of plasmin cleaved fibrin α chain (DNTYNRVSED), β chain (DNENVVNEYS) and γ chain (AIQLTYNPDE). (B) Antibody reactivity towards peptides selection (DNENVVNEYS), truncated (NENVVNEYS) and elongated (KDENVVNEYS). (C) Competitive ELISA specificity on fibrinogen and fibrin degradation products. (D) Sandwich ELISA specificity on fibrin degradation products.

164'DNENVVNEYS'173 and fibrinogen/fibrin cleavage product by plasmin (Fig. 2A, C), but did not recognize N-terminus of plasmin cleaved fibrin α chain DNTYNRVSED, plasmin cleaved fibrin γ chain AIQLTYNPDE, elongated peptide KDENVVNEYS, truncated peptide NENVVNEYS or total fibrinogen (Fig. 2A, B, C). When 3A9 was applied in a sandwich ELISA (Fig. 1B), it only measured cross-linked fibrin degradation products, but not fibrinogen degradation products (Fig. 2D). The data confirmed that the competitive ELISA (D-fragment) can measure fibrinogen/fibrin degradation products, while the sandwich ELISA (D-dimer) only measured cross-linked fibrin degradation products.

Technical performance of D-fragment competitive assay and D-dimer sandwich assay

The measurement range of the D-fragment and D-dimer assays was determined by LLOD and ULOD, providing a range from 54.2-3309.4 ng/ml and 4.9-487 ng/ml, respectively. The average intra- and inter-assay variations of both assays were

below 10% and 20%, respectively. The dilution and spiking recovery of human plasma and serum were within $100\pm 20\%$. The freeze-thaw recovery was within $100\pm 20\%$. The assays have no interference with HAMA, biotin, hemoglobin and intralipid. All technical performances are listed in Table II. The data confirmed technically robustness of the assays.

FPA, D-fragment and D-dimer levels in CD study and healthy controls

The fibrin formation marker, FPA, was significantly higher in non-IBD and CD patients than in healthy controls ($p < 0.0001$, Fig. 3A). For the fibrinogen/fibrin degradation products (measured by D-fragment assay), there was a trend of lower levels in non-IBD and CD patients, although no significant differences were observed when compared to healthy controls (Fig. 3B). For the cross-linked fibrin degradation product (measured by D-dimer assay), the level was significantly lower in CD patients compared to healthy controls ($p = 0.0001$) (Fig. 3C). The ratio between D-fragment and D-dimer was higher

Table II. Technical performance test in D-fragment competitive and D-dimer sandwich assays

Technical test	D-fragment assay	D-dimer assay
LLOD	54.2 ng/ml	4.9 ng/ml
ULOD	3309.4 ng/ml	487 ng/ml
Dilution recovery	$100\pm 20\%$ (EDTA-plasma and serum)	$100\pm 20\%$ (EDTA- and citrate-plasma and serum)
Spiking recovery	$100\pm 20\%$ (EDTA-plasma and serum)	$100\pm 20\%$ (EDTA- and citrate-plasma and serum)
Intra assay variation	7.7%	5.5%
Inter assay variation	17.8%	14.8%
Interference		
(HAMA, Biotin, hemoglobin, intralipid)	No	No
Sample freeze-thaw recovery (4 cycles)	$100\pm 20\%$ (EDTA-plasma and serum)	$100\pm 20\%$ (EDTA-plasma and serum)

LLOD: lower limit of detection; ULOD: upper limit of detection

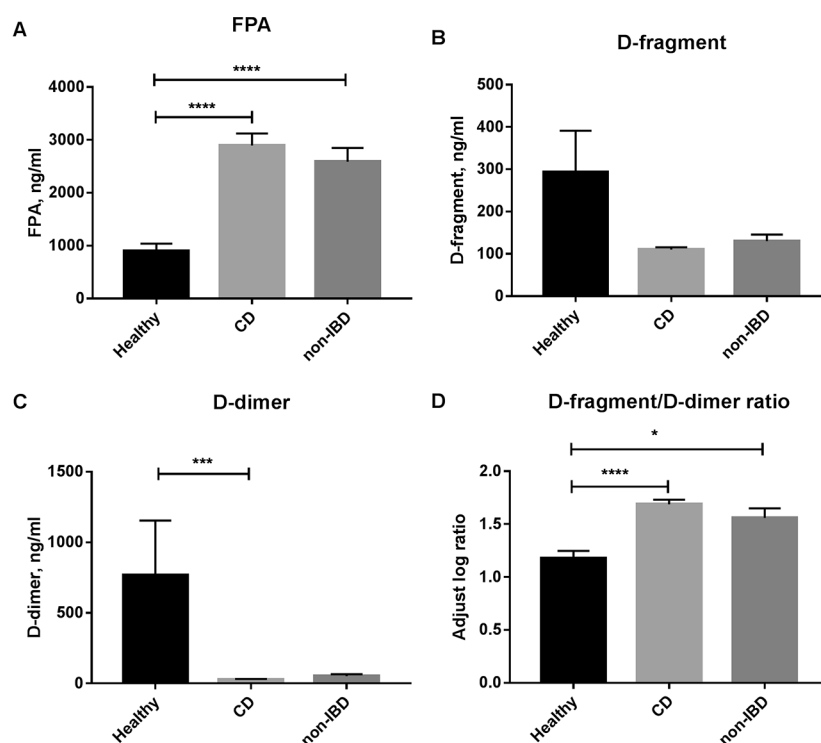


Fig. 3. FPA, D-fragment and D-dimer levels in IBD clinical study and healthy donor controls. (A) FPA. (B) D-fragment. (C) D-dimer. (D) D-fragment/D-dimer ratio. All graphs presented means \pm SEM. Differences between control subjects, non-IBD and CD patients were considered statistically significant if $p < 0.05$ and significance levels were displayed as: *= $p < 0.05$; **= $p < 0.01$, ***= $p < 0.001$, and ****= $p < 0.0001$.

in CD and non-IBD patients than in healthy controls ($p < 0.0001$ and $p = 0.0211$, Fig. 3D). D-fragment and D-dimer levels were strongly correlated (Spearman $r = 0.403$, $p = 0.00017$). D-dimer was correlated with disease parameter CDAI (Spearman $r = 0.427$, $p = 0.017$). There is also a trend of higher D-dimer levels in active CD patients (CDAI > 150) than in inactive CD patients (CDAI < 150), median 29.52 ng/ml vs. 14.74 ng/ml, with no statistical significance ($p = 0.10$). The other biomarkers did not correlate with CDAI or C-reactive protein (CRP).

The diagnostic value of FPA and D-dimer in the CD study and healthy controls

To differentiate non-IBD patients from healthy controls, FPA, D-dimer and D-fragment/D-dimer ratio had AUC=0.94 ($p < 0.0001$), 0.68 ($p = 0.0374$) and 0.75 ($p = 0.0014$), respectively. To differentiate CD patients from healthy controls, FPA, D-dimer and D-fragment/D-dimer ratio had AUC=0.92 ($p < 0.0001$), 0.78 ($p < 0.0001$) and 0.85 ($p < 0.0001$) respectively. However, FPA, D-dimer and D-fragment/D-dimer ratio did

Table III. AUC ROC curve analysis of FPA and D-dimer

	AUC	95% CI	Sensitivity; Specificity	P-value
CD vs. healthy				
FPA	0.92	0.83 to 0.97	91.2; 91.4	<0.0001
D-dimer	0.78	0.67 to 0.87	84.6; 61.3	<0.0001
D-fragment/D-dimer	0.85	0.75 to 0.93	84.6; 83.3	<0.0001
Non-IBD vs. healthy				
FPA	0.94	0.83 to 0.99	91.2; 93.3	<0.0001
D-dimer	0.68	0.54 to 0.81	89.7; 46.2	0.0374
D-fragment/D-dimer	0.75	0.61 to 0.86	84.6; 69.2	0.0014
CD vs. Non-IBD				
FPA	0.57	0.43 to 0.71	66.7; 62.9	0.40
D-dimer	0.62	0.46 to 0.76	38.5; 87.1	0.23
D-fragment/D-dimer	0.61	0.45 to 0.75	30.8; 96.7	0.28

not have enough diagnostic power to differentiate CD patients from non-IBD patients (Table III). D-fragment did not show enough diagnostic power to be included.

DISCUSSION

We hypothesized that the process of wound healing in CD patients was compromised, which would be reflected in fibrinogen turnover. To investigate this, we measured newly developed serological biomarkers of clot formation and fibrinolysis in a cohort of CD patients together with age-matched control subjects. The competitive D-fragment assay detects both fibrinogen and fibrin degradation products, which represents the total clot resolution. The sandwich D-dimer only detects fibrin degradation, which represents the cross-linked fibrin by factor XIII clot resolution. To our knowledge, this is the first study demonstrating that clot formation and clot resolution were impaired in CD patients due to increased fibrin synthesis but with compromised cross-linked fibrin degradation.

One of the main findings was that FPA levels were significantly increased in inflammatory conditions of non-IBD and CD patients, indicating increased ongoing wound healing and clot formation as compared to healthy individuals. We also found that D-dimer levels were significantly decreased in CD patients, suggesting decreased cross-linked fibrin resolution. The fact that the ratio of D-fragment/D-dimer was increased in CD and non-IBD patients, while the D-fragment was not changed, suggests the cross-linked fibrin degradation was compromised in terms of all fibrinogen/fibrin degradation. Finally, we also demonstrated that FPA, D-dimer, and D-fragment/D-dimer ratio had a diagnostic power of AUC=0.92, 0.78 and 0.85, respectively, to differentiate CD patients from healthy controls.

Biomarkers of clot formation and fibrinolysis

The competitive FPA assay quantifies clot formation, as FPA is cleaved from fibrinogen, when soluble fibrinogen in the serum is converted into gel-forming fibrin at the wound site. Fibrinogen is a large glycoprotein and is composed of two sets of α , β and γ chains which bind together by disulfide bonds, with molecular weight around 340kD [16,17]. The N-terminal fibrinopeptides of α chain (FPA) and β chain (FPB) are released by thrombin and initiate the fibrin conversion [18]. FPA serum levels reflect the "storage level" of fibrinogen, which will convert to fibrin-rich clot when injuries happen.

After fibrinogen is converted into fibrin, fibrin monomers are cross-linked by FXIIIa to ensure a stable clot formation at the wound site. Under catalyzing of FXIIIa, the C-terminal region of γ fibrin-chains is cross-linked by covalent bonds through K'406 in one γ chain and E'398/399 in the other γ fibrin-chain [16]. Crosslinks between α - α fibrin chains or α - γ fibrin chains are also observed [19,20]. During degradation, fibrinogen can be cleaved by plasmin and generates terminal products fragment D and E (Fig 1A) [21]. Since the crosslinks by themselves are resistant to plasmin cleavage, the cross-linked fibrin generates terminal product D-dimer and also intermediate polymers (Fig 1B) [22, 23]. The 3A9 monoclonal antibody was applied in two distinct ELISAs, a) a sandwich D-dimer assay b) a competitive D-fragment assay, which

enabled us to separate stable clot resolution from total clot resolution, respectively. We measured the exact plasmin cleavage within fibrin, which reflects the amount of plasmin cleavage during cross-linked clot resolution.

D-dimers have been studied for over 40 years. Up to date, there are over 20 commercialized D-dimer tests, e.g VIDAS® D-Dimer Exclusion (BioMérieux), Alere Triage® D-Dimer Test (Alere), STA®-Liatest® D-Di Plus (Stago), Tina-quant D-Dimer (Roche) etc. They all use monoclonal antibodies, which recognize the epitope from D-dimer (fibrin) and show no reaction to cross-linked fibrin or fibrinogen. However, only a few antibodies have clearly determined epitopes. For example, the first generation of D-dimer antibody DD-3B6 recognizes a conformation change in D-dimer. The epitope is within γ chain amino acid 86-302, which only is exposed after plasmin degradation of cross-linked fibrin [24]. Antibody J1F-23 recognizes the newly exposed N-terminus of γ chain 63-85 [25]. Antibody B4 recognizes the beta chain 134-142 and weakly recognizes alpha chain 124-214 [26]. In 2001, a study investigated the reactivity to fibrin/fibrinogen degradation products in 21 commercial D-dimer tests [27]. Some of the commercial kits have obvious cross-reaction to fibrinogen degradation products, which will somehow affect the accuracy. Therefore, we developed a more specific D-dimer sandwich ELISA, targeted specifically to the plasmin cleavage site in fibrin β chain and the cross-linked fibrin degradation products.

Altered turnover of fibrinogen in CD patients

Crohn's disease is characterized by repeated epithelial and endothelial damage. Continuous and sufficient wound healing with an optimal balance between clot formation and clot resolution is required to repair the injury. Our data showed that CD patients were prone to start more fibrin formation but had impaired fibrinolysis. Up-regulated FPA levels in CD patients suggested increased clot formation with more fibrinogen to convert into fibrin during injury (enhanced pre-wound healing). This was observed for non-IBD as well (the patient group with high CRP levels) confirming that under increased inflammatory conditions, clot formation was increased. The D-dimer results, however, showed decreased D-dimer levels in CD patients only, suggesting that fibrinolysis was impaired in CD patients due to decreased capacity of fibrin degradation or insufficient cross-linked fibrin. The FPA, D-dimer, and D-fragment/D-dimer ratio had a diagnostic power of AUC=0.92, 0.78 and 0.85 respectively to differentiate CD patients from healthy controls and they may be beneficial as biomarkers to assess the health status of CD patients.

A few studies have investigated D-dimer levels in CD patients. Most of the studies have shown that D-dimer levels were the same/increased in CD patients compared with healthy controls [9, 11, 28]. Furthermore, some studies found D-dimer level was higher in patients with active disease compared with inactive disease [7, 29], demonstrating that D-dimer was related to the disease status. However, our study found that the D-dimer levels were actually decreased in CD patients. This key finding may be caused by targeting the novel neo-epitope. Notably, the neo-epitope D-dimer antibody we applied, entails a different epitope than other assays. The antibody was not

targeting a conformation change but targeting the plasmin cleavage in the β chain. To our knowledge, this assay is the only one that detects this neo-epitope.

In our study, the turnover of fibrinogen/fibrin was clearly unbalanced in CD patients, which may result in fibrin deposition in the intestinal injury site. In direct alignment, FXIIIa reduction has been observed in CD patients [7, 8], where it was suggested that although FXIIIa might be rapidly consumed [7], the cross-linking of fibrin may not be properly formed and might contribute to the damage of mucosal lining. The altered fibrin deposition, a consequence of impaired fibrinolysis and clot structure alteration by compromised fibrin cross-linking, is part of the pathological background of CD and UC patients having an increased risk of thrombosis [30, 31].

We acknowledge that there are some limitations in this study. The healthy controls were obtained from a commercial company, as compared to the disease affected sample, albeit sample storage and sample age was properly controlled. The number of patients and healthy controls was relatively low. Consequently, we need to validate these findings in a larger study. Endoscopy and image examinations are not available for this cohort. The future studies should focus on evaluating the biomarkers in terms of endoscopic and histological mucosal healing.

CONCLUSIONS

We found that CD patients have higher levels of the fibrin formation marker (FPA), but lower levels of the fibrinogen/fibrin degradation marker (D-fragment and D-dimer). The overall turnover of fibrin was unbalanced, which suggested an impaired wound healing in CD patients. The novel assessment of the turnover of fibrinogen/fibrin components provided insights suggesting that the fibrinolysis system was impaired in CD, with an altered quality suggesting this to be an integral part of the disease representation.

Conflicts of interest: TMJ, SS, YYL, JHM, ACBJ and MAK are employed by Nordic Bioscience, Biomarkers and Research. MAK, ACBJ and TMJ hold stock in Nordic Bioscience. Nordic Bioscience is a privately owned small-medium sized enterprise, partly focused on the development of biomarkers for connective tissue disorders and rheumatic diseases. None of the authors received any kind of financial benefits or other bonuses for the work described in this manuscript.

Authors' contributions: Conception and design: TMJ, MAK. Analysis and interpretation of the data: SS. Antibody development: YYL. Providing clinical samples: JK, AK, MDJ. Drafting of the article: SS, TMJ. Critical revision of the article for important intellectual content: MAK, JHM, JK, AK, MDJ, ACBJ. All the authors approved the final version of the manuscript.

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