

## **Initiation of the Wound Healing Cascade in Inflammatory Bowel Disease: Assessment of Von Willebrand Factor ADAMTS-13 Processing and Formation in Crohn's Disease**

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### **Abstract**

**Background:** Crohn's disease (CD) is a chronic inflammatory condition of the intestinal tract resulting in severe intestinal tissue damage. The concept of mucosal healing has therefore gained significant interest as a therapeutic end point in the treatment of inflammatory bowel disease (IBD). Given that the function of von Willebrand factor (VWF) is a central axis in initiation of wound healing and a marker of endothelial dysfunction, we hypothesized that changes in processing of VWF may provide additional disease characterization of CD.

**Methods:** We developed neo-specific biomarkers specifically targeting the ADAMTS13-processed form of VWF (VWF-A), and formation of VWF by quantification of the pro-peptide (VWF-N). We measured these two analytes of VWF in 71 serum samples from diseased patients of which 51 were classified as CD patients and 20 as non-IBD patients. 10 age-matched healthy serum samples were included as control group.

**Results:** Levels of VWF-A ( $p < 0.01$ ) and VWF-N ( $p < 0.0001$ ) were significantly increased in CD and non-IBD patients compared to healthy control subjects. VWF-N level ( $p < 0.001$ ) discriminated CD and non-IBD patients from control subjects with a diagnostic accuracy of 94%. No significant differences were observed in the turnover ratio of VWF-N/VWF-A between CD, non-IBD and healthy subjects.

**Conclusion:** Patients with CD have increased production of VWF and ADAMTS13 proteolyzed-form of VWF compared to healthy subjects. Biomarkers of primary wound healing could serve as early predictors of disease activity and intestinal healing in patients with CD and may be used to assess personalized health status and treatment options for patients.

**Keywords:** *Endothelial Injury; ADAMTS13; Von Willebrand Factor; Crohn's*

### **Abbreviations**

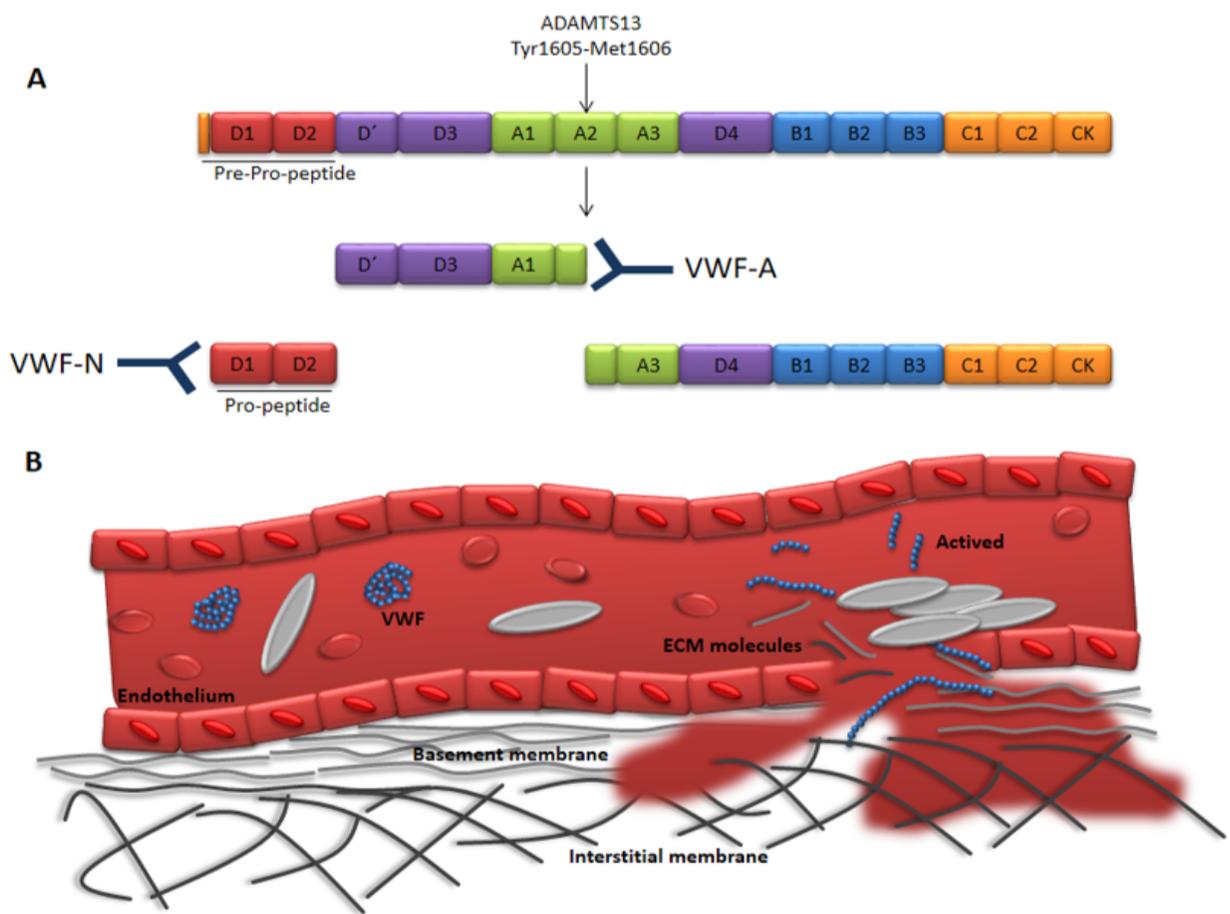
ADAMTS13: Disintegrin and Metalloproteinase with Thrombospondin Type 1 Motif, Member 13; AUC: Area Under the Receiver-Operating Characteristic Curve; CD: Crohn's Disease; CRP: C-reactive Protein; ECM: Extracellular Matrix; HAMA: Human Anti-Mouse Antibody; IBD: Inflammatory Bowel Disease; LLOD: Lower Limit of Detection; MMP: Matrix Metalloproteinase; rVWF-A2: Recombinant VWF Protein; SEM: Standard Error of Mean; TMB: Tetramethylbenzidine; TTP: Thrombotic Thrombocytopenic Purpura; ULOD: Upper Limit of Detection; ULVWF: Ultra Large VWF Multimers; VWD: Von Willebrand Disease (VWD); VWF: Von Willebrand Factor; VWF-A: ADAMTS13-processed Form of VWF; VWF-N: Formation of VWF

**Introduction**

Crohn's disease (CD) is a chronic inflammatory condition of the gastrointestinal tract resulting in severe intestinal tissue damage [1]. In general, there is a consensus in the field that mucosal healing is a valid endpoint for evaluating efficacy of IBD treatments [2,3]. Unfortunately, mucosal healing assessment is presently tedious, requiring colonoscopy and histopathological assessment, which is costly and impractical [3,4]. By assessing the wound healing processes, a better understanding of mucosal healing and how it can be compromised in CD, may prove to be valuable to evaluate health status and treatment options for patients [5,6].

Von Willebrand factor (VWF) is a multimeric glycoprotein with critical functions for wound healing. Key to VWF function is mediating platelet adhesion and platelet aggregation to the damaged endothelium, by providing a link between the exposed subendothelial matrix and platelets [7]. VWF is stored in Weibel-Palade bodies of endothelial cells as well in  $\alpha$ -granules of platelets and is secreted as ultra large VWF multimers (ULVWF) leading to recruitment of platelets and leukocytes. The highly adhesive ULVWF can be proteolytically processed by a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 (ADAMTS13) [8] releasing smaller and less bioactive VWF multimers into the blood circulation. Thus, ADAMTS13-mediated cleavage of VWF is important for removing adherent VWF from the endothelium and reducing platelet tethering and leukocyte recruitment [9-11]. Quantitative and/or qualitative malfunctions of VWF are associated with impaired wound healing causing severe bleeding tendency or thrombosis, as seen with von Willebrand disease (VWD) [12,13] and thrombotic thrombocytopenic purpura (TTP), respectively [14,15].

Given that VWF is a central axis in the initiation of wound healing and maintains the hemostatic balance, as well as being a marker of endothelial dysfunction, we hypothesized changes in processing of VWF by targeting neo-epitopes fragments of VWF that are released during tissue repair, may provide additional disease characterization of CD. Any dysregulation of VWF processing could contribute to the development and progression of CD by expanding the intestinal microvascular and sustaining the inflammatory responses. Increased angiogenesis, leukocyte extravasation and increased coagulation may generate a hypercoagulable state followed by prothrombotic conditions as seen in some patients with IBD [16-18]. We developed two neo-epitopes competitive ELISAs, detecting the synthesis and ADAMTS13 proteolyzed-form of VWF, named VWF-N assay and VWF-A assay, respectively. Quantifying both formation of VWF and ADAMTS13-mediated degradation of VWF enabled us to investigate VWF processing which may add distinct information to the pathogenesis of the disease.



**Figure 1:** Schematic overview of VWF protein and function. A) The mature VWF protein is divided into series of domains where proteolytic cleavage site for ADAMTS13 is located within the A2 domain. The neo-epitope antibodies VWF-N (15D2) and VWF-A (7G1) targets the pro-peptide and ADAMTS13-released VWF-A fragment, respectively. B) VWF is initially synthesized as a series of very large multimers that upon shear stress is degraded by the metalloprotease ADAMTS13 that regulates VWF multimeric size and platelet-tethering function.

## Materials and Methods

### IBD clinical study

This cohort is part of a prospective blinded multicenter study from Odense University Hospital termed the "CD study" evaluating three small bowel imaging techniques (ClinicalTrials.gov Identifier NCT01019460) [5,19]. Out of the original 104 serum samples, we had access to the remaining 71 of which 51 were diagnosed with CD and 20 with non-IBD gastrointestinal diseases. As control group, we included 10 age-matched healthy serum samples from a commercial company (Valley Biomedical). The healthy donors showed no signs of illness by standard medical assessment and were found to be negative for hepatitis B virus s-antigen, hepatitis C virus, human immunodeficiency virus-1, human immunodeficiency virus-2, human immunodeficiency virus-1 antigen, human immunodeficiency virus-1 nucleic acid testing and syphilis by FDA-Approved Methods. Informed consent had been collected from all participants and in accordance with Danish law, no additional ethical approval for measuring the biomarkers was acquired for this study, since ethical approval for measuring biomarkers in previously collected samples is not required.

### Animals

We used six week-old BALB/cOlaHsd female mice (ENVIGO). Mice were housed under standardized conditions (20 - 23°C, 30 - 60% relative humidity, and a 12-h/12-h light/dark cycle) and were caged five mice per cage in cage type type IIIH (1291H, 425 x 266 x 185 mm, floor area 800 cm<sup>2</sup>) from Scanbur DK. They had access to food and water ad libitum, and the general conditions were monitored daily by trained animal technicians. To minimize the stress, the mice were only handled when necessary during dosing and when changing the cages. The mice also had access to materials for nest-building and lay calmly by having access to Enviro-DRI (31008), mouse house or igloo (Scanbur), Tapvai 2HV (37311), Aspe wooden stick S (30968) and NESTLETS (31007) from Brogaarden DK. When the analgesic is given, it was only by trained animal technicians. Mice were sacrificed by cervical dislocation. The study was approved by the Danish Animal Experimentation council (J. Nr.: 2013-15-2934-00956) and conducted according to national legislation.

### Monoclonal neo-specific antibody development for VWF-A and VWF-N

The sequences <sup>1596</sup>-DREQAPNLVY-<sup>1605</sup> and <sup>23</sup>-AEGTRGRSST-<sup>32</sup> (American Peptide Company, USA) as the immunogenic peptide were used to generate specific neo-epitope monoclonal antibodies for VWF-A and VWF-N respectively. The mice were immunized subcutaneously with 200 µl Freund's incomplete Adjuvant (F5506, 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 the highest antiserum titer and the best native reactivity was selected for fusion. The selected mouse was boosted intraperitoneally with 50 µg of immunogenic peptide in 100 µl 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion. The fusion procedure has been described elsewhere [20]. 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 CO<sub>2</sub>-incubator. Here standard limited dilution was used to promote monoclonal growth. Clones specific to the standard peptide and without cross-reactivity to neither elongated peptides (DREQAPNLVYM and CAEGTRGRSST, American Peptide Company, USA) nor truncated peptides (DREQAPNLV and EGTRGRSST, American Peptide Company, USA) for VWF-A and VWF-N respectively, were selected and sub-cloned. Finally, the antibodies were purified using an IgG column.

### VWF-A and VWF-N ELISA assay protocol

ELISA-plates used for the assay development were Streptavidin-coated from Roche (cat.: 11940279). All ELISA plates were analyzed with the ELISA reader from Molecular Devices, SpectraMax M (CA, USA). A 96-well streptavidin plate was coated with biotinylated synthetic peptide-biotin-DREQAPNLVY or biotin-AEGTRGRSST for VWF-A or VWF-N respectively (American Peptide Company, USA), dissolved in coating buffer (40 mM Na<sub>2</sub>HPO<sub>4</sub>, 7 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 1% BSA, pH 7.4) and incubated 30 minutes at 20°C. 20 µL of standard peptide or samples diluted in incubation buffer (40 mM Na<sub>2</sub>HPO<sub>4</sub>, 7 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 1% BSA, pH 7.4) were added to appropriate wells, followed by 100 µL of monoclonal antibody 7G1 or 15D2, VWF-A or VWF-N respectively, and incubated 20 hours at 4°C. For the VWF-A assay a HAMA blocker (5% liquid II) was included in the incubation buffer. After plate wash, 100 µL of Horseradish Peroxidase labeled rabbit anti-mouse Ab (315-035-045, Jackson Immuno Research) was added and incubated at 20°C for 1 hour. Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec cat.4380H) was added and the plate

was incubated 15 minutes at 20°C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl). The TMB reaction was stopped by adding 100 µL of stopping solution (1% H<sub>2</sub>SO<sub>4</sub>) and measured at 450 nm with 650 nm as the reference.

#### **Technical evaluation of VWF-A and VWF-N assays**

The lower limit of detection (LLOD) was determined from 21 zero samples (i.e. buffer) and calculated as the mean + 3x standard deviation. Upper limit of detection (ULOD) was determined as the mean - 3xSD of 10 measurements of Standard A. The intra-assay and inter-assay variations were the mean variations of 7 QC samples run 10 independent times in duplicate. Dilution recovery was determined in 3 plasma and serum samples (Innovative Research) and was calculated as a percentage of recovery of diluted samples from the 100% sample. Spike recovery was calculated by comparing different concentrations of peptide solution in buffer and in human plasma samples (Valley Biomedical). Interference by hemoglobin, lipemia, biotin, and human anti-mouse antibody (HAMA) was determined in 90 ng/ml Biotin, 5 mg/ml hemoglobin, 5 mg/ml intralipid and high level of HAMA samples. Analyte stability was determined for three healthy human citrate plasma samples for four freeze/thaw cycles and calculated as the percentage recovery of the first freeze-thaw cycle. Same samples were tested at 2 hrs, 4 hrs and 24 hrs at 4°C and 20°C against non-stressed analytes. Recovery percentage was calculated with the control samples as a reference and accepted within 100% recovery ± 20%.

#### **In vitro cleavage of VWF protein**

The *in vitro* cleavage of recombinant VWF protein (reVWF-A2) was performed according to the manufacturer's instruction (R&D system). Briefly, 25 µL of 200 µg/mL reVWF-A2 protein (*E. coli*-derived Asp1498-Val1665, 2764-WF, R&D system) was mixed with 25 µL of 20 µg/mL ADAMTS13 (6156-AD, R&D system). The mixture was incubated at 37°C for 2 hours. The reaction was terminated by adding 2.6 µL 200 mM EDTA.

#### **Western blotting**

ReVWF-A2 and ADAMTS13 cleaved VWF-A2 were separated by SDS-PAGE, and proteins were transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk powder in TBS-T buffer and incubated at room temperature for two hours. The membranes were incubated with primary antibody at 4°C overnight, followed by three times wash in TBS-T buffer. Subsequently the membranes were incubated in the secondary peroxidase conjugated antibody and followed by three times wash. Finally, the results were visualized with ECL detection system (cat# RPN2109, Amersham Pharmacia). The commercial antibody (MAB27641, R&D system) recognizes both total and degraded fragment of VWF-A2.

#### **Statistics**

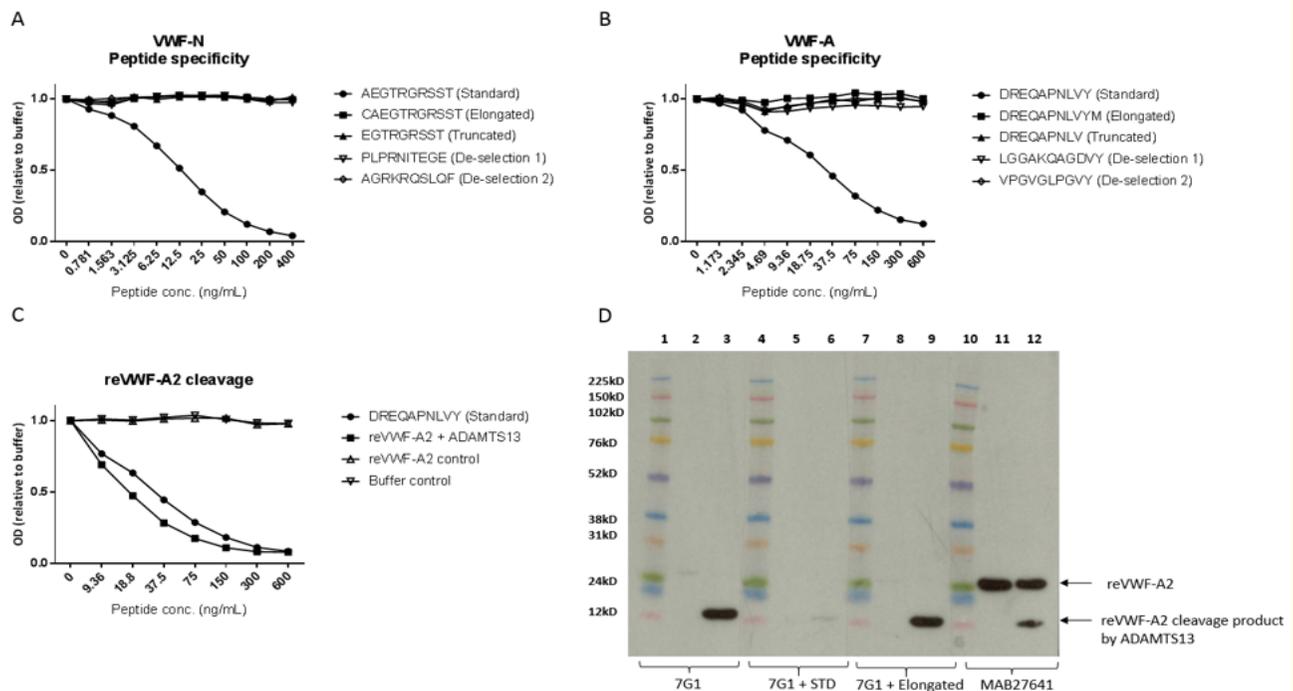
The biomarker data were not normally distributed (D'agostino Pearson test), thus for comparison, all data were analyzed as non-parametric (Mann-Whitney test) and by using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Significance threshold was set at  $p < 0.05$ . To investigate the turnover of VWF, data were logarithmically transformed to reduce or eliminate data skewness. The area under the receiver-operating characteristics (ROC) curve (AUC) was estimated and confounding factors and correlations were investigated using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2014).

## **Results**

#### **Characterization of VWF assays**

The chosen monoclonal 15D2 antibody for the VWF-N assay recognized the N-terminal part of the pro-peptide, the 10 amino acid sequence <sup>23</sup>AEGTRGRSST<sup>32</sup>, but not the elongated peptide CAEGTRGRSST or the truncated EGTRGRSST peptide sequence. These results verify the neo-specificity of the antibody (Figure 2B). The chosen monoclonal 7G1 antibody for the VWF-A assay specifically recognized

the C-terminal 10 amino acids <sup>1596</sup>DREQAPNLVY<sup>1605</sup> of the N-terminal fragment of mature VWF following ADAMTS13 cleavage, but did not recognize the elongated peptide DREQAPNLVYM, truncated peptide DREQAPNLV or un-cleaved VWF-A2 protein (Figure 2A and 2C). These results were confirmed by western blotting of *in vitro* ADAMTS13 cleaved reVWF-A2. The antibody 7G1 only recognized the 12 kDa cleaved fragment, but not the 25 kDa total reVWF-A2 protein (Figure 2D). Both bands were detected by the antibody recognizing the total reVWF-A2 (Figure 2D). To ensure the specificity of the blot, primary antibody incubation was done including the standard and elongated peptide. The signal was completely blocked in the presence of the standard peptide, but not with the elongated peptide, confirming the neo-specificity of the antibody (Figure 2D).



**Figure 2:** Specific reactivity of the 7G1 and 15D2 antibody towards VWF-A and VWF-N peptide, respectively. Competitive ELISA against standard-, elongated-, truncated- and two de-selection-peptides of selected monoclonal antibody A) VWF-N (15D2) and B) VWF-A (7G1), presented as relative OD values as compared to buffer controls as a function of peptide concentration (buffers set to OD 1.0). C) Competitive ELISA against recombinant VWF-A2 protein (reVWF-A2) and reVWF-A2 cleavage product by ADAMTS13. Standard peptide and buffer included as positive and negative controls, respectively. Peptide concentration is that of the standard peptide. Concentration of reVWF-A2 was a 2-fold dilution starting at 2500 ng/ml. D) Western blot of cleavage material.

### Technical performance of the VWF assays

The measurement range of the VWF-A competitive ELISA was determined by LLOD and ULOD providing a range from 2.1 ng/ml to 406.1 ng/ml. The assay had an average intra- and inter-assay variation of 3.5% and 10.0%, respectively. The measuring range of the VWF-N assay was 4.3 ng/mL (LLOD) to 331.6 ng/mL (ULOD) with an intra- and inter-variation of 2.7% and 9.2%, respectively. Both the VWF-A and VWF-N showed dilution and spiking recovery in human serum as well as human plasma EDTA, plasma heparin and plasma EDTA

within the accepted  $100 \pm 20\%$  range (Table 1). No interference with hemoglobin, intralipid or biotin was observed in either VWF assay. Analytic stability and freeze-thaw cycles were within the accepted  $100 \pm 20\%$  range (data not shown). Overall, both VWF assays showed good technical performance with robustness for all tested matrices.

	<b>CD (n = 51)</b>	<b>Non-IBD (n = 20)</b>
<b>Gender</b>		
Female, (%)	20 (39 %)	5 (25 %)
Male, (%)	31 (61 %)	15 (75 %)
Age Mean; [range]	34.8 years [15 - 72]	34.5 years [15 - 75]
<b>Use of corticosteroids</b>		
Yes (%)	28 (55 %)	1 (5 %)
No (%)	23 (45 %)	19 (95 %)
<b>Disease activity (CDAI score)</b>		
Active disease (>150) (%)	23 (49 %)	N/A
Remission (>150) (%)	24 (51 %)	N/A
<b>Montreal classification</b>		
<b>Behaviour</b>		
B1	20	N/A
B2	14	
B3	17	
<b>C-reactive protein</b>		
< 5 mg/L	27	11
> 5 mg/L	24	9

**Table 1:** Patients' demographics.

<b>Measurements</b>	<b>Technical characteristics</b>	
	<b>VWF-A</b>	<b>VWF-N</b>
Lower limit of detection (LLOD)	2.1 ng/ml	4.3 ng/ml
Upper limit of detection (ULOD)	406.1 ng/ml	331.6 ng/ml
Intra-assay variability (accepted < 10%)	3.5%	2.7%
Inter-assay variability (accepted < 15%)	10.0%	9.2%
Dilution recovery (plasma EDTA, Citrate, heparin, serum)	Within $100 \pm 20\%$	Within $100 \pm 20\%$
Spiking recovery (plasma EDTA, Citrate, heparin, serum)	Within $100 \pm 20\%$	Within $100 \pm 20\%$
Interference (Hgb, lipid, Biotin),	No interference	No interference, no HAMA interference

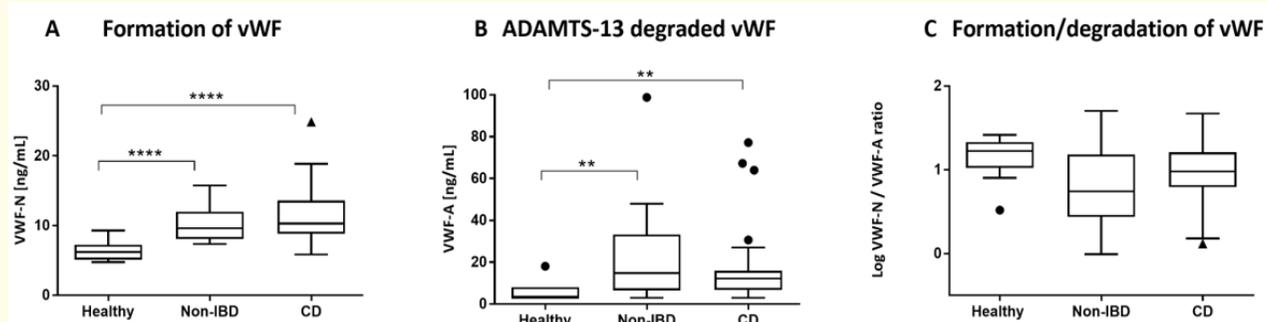
**Table 2:** Technical performances of VWF-A and VWF-N assays.

Hgb: Hemoglobin; Biotin (Vitamin H); HAMA: Human Anti-Mouse Antibody.

### VWF-A and VWF-N in Crohn's disease

We tested the VWF-A and VWF-N biomarkers in a cohort from a CD study including 71 serum samples in total, of which 51 were classified as CD and 20 as non-IBD patients. We included 10 healthy samples as a non-disease control. Concentrations of VWF-A ( $p < 0.01$ ) and VWF-N ( $p < 0.0001$ ) were significantly increased in both CD and non-IBD patients compared to healthy controls, while there was no significant difference in concentrations between the two disease-groups irrespective of the VWF form (Figure 3).

To address whether there was a significant difference in the processing of VWF between the patient groups, we looked at the log-ratio between synthesized VWF (VWF-N) and hemostasis-activated VWF (VWF-A). We found no significant change in the distribution or processing of the VWF forms between the patient groups, meaning that both VWF-A and VWF-N levels were increased in CD and non-IBD patients with no significant change in the ratio of the VWF forms within the three groups (Figure 3C). We subsequently estimate the discriminative power of the VWF markers by differentiating healthy patients from CD or non-IBD patients. VWF-N had the highest diagnostic power to differentiate healthy patients from CD and healthy from non-IBD, with an area under the curve (AUC) of 0.94 ( $p < 0.0001$ ) in both cases (Table 3). VWF-A differentiated healthy subjects from CD and non-IBD with the AUC being 0.80 ( $p = 0.0001$ ) and 0.82 ( $p < 0.0001$ ) respectively. Neither of the VWF markers showed good discriminating power when looking to differentiate CD from non-IBD patients, with an AUC of 0.60 ( $p = 0.206$ ) for VWF-A and 0.61 ( $p = 0.123$ ) for VWF-N. Overall the two markers showed higher VWF levels during disease and a good separating power to discriminate healthy subjects from both CD and non-IBD patients.



**Figure 3:** Formation and activation of VWF is increased in IBD patients compared to control subject. A: VWF-A B: VWF-N C: mean log ratio of VWF-N/VWF-A adjusted so a ratio of 1 indicates equal biomarker levels. Data were analyzed using Mann-Whitney test and presented as a Tukey box plot. Differences between control subjects, non-IBD and CD patients were considered statistically significant if  $p < 0.05$  and significance levels are displayed as: \*\*,  $p \leq 0.01$ . \*\*\*\*,  $p \leq 0.0001$ .

	AUC [95% CI]	Sensitivity; specificity % (Youden index)	p-Value
<b>Biomarker: CD vs. Healthy</b>			
VWF-A	0.80 [0.68 - 0.89]	72.6; 90.0	0.0001
VWF-N	0.94 [0.84 - 0.98]	90.2; 90.0	< 0.0001
<b>Biomarker: Non-IBD vs healthy</b>			
VWF-A	0.82 [0.63 - 0.93]	70.0; 90.0	< 0.0001
VWF-N	0.94 [0.79 - 0.99]	80.0; 100.0	< 0.0001
<b>Biomarker: CD vs non-IBD</b>			
VWF-A	0.61 [0.48 - 0.72]	94.1; 35.0	0.206
VWF-N	0.61 [0.49 - 0.72]	88.2; 35.0	0.137

**Table 3:** AUC ROC curve analysis of the individual biomarkers.

AUC: Areas under the Curve; CI: Confidence Interval; CD: Crohn's Disease; IBD; Inflammatory Bowel Disease.

## Discussion

We investigated the processing of VWF, a marker of endothelial dysfunction, by measuring two analytes of VWF using newly developed serological biomarkers targeting neo-epitopes of synthesized VWF (VWF-N) and ADAMTS13 proteolyzed-form of VWF (VWF-A). Assessing neo-epitope protein fragments of VWF might add additional information to the pathogenesis of Crohn's disease, which is not available when evaluating intact VWF. Neoepitopes have been proven to be more accurate predictors of disease than their unmodified intact protein of origin [21,22], since measurement of fragments from the same protein has yielded distinct information [23,24]. We found significantly increased levels of VWF-N and VWF-A in CD and non-IBD patients compared to healthy subjects. These significant differences between diseased and healthy controls were further substantiated when VWF-N was found to discriminate CD and non-IBD patients from control subjects with a diagnostic accuracy of 94% in both cases. In addition, the ratio of VWF-N/VWF-A was not altered between subjects/patient groups indicating a normal turnover of VWF in intestinal Crohn's.

## Biomarkers of VWF

We described the development of two competitive ELISA assays addressing the processing of VWF. The VWF-N assay measured synthesized VWF by targeting the neo-epitope of the N-terminal part of the pro-peptide. VWF is synthesized as a pre-pro-peptide and upon secretion from Weibel-Palade bodies of endothelial cells mainly, the pro-peptide is separated from the mature VWF [25,26]. The pro-peptide and mature protein of VWF exist in a 1:1 ratio and following dissociation the pro-peptide has an estimated half-life of 2h [27,28]. Other commercialized available assays do exist that determine the level of pro-peptide VWF, but not neo-specific and they are historically known as VWFpp or VWF:AgII.

Plasma metalloproteinase ADAMTS13 is the only protease we know of that regulates VWF multimer sizes by cleaving ULVWF into smaller and less thrombogenic fragments, which can bind to a variety of extracellular matrix components and platelets to assist in blood clot formation [8,10,29]. Our VWF-A assay addresses ADAMTS13 proteolyzed-form of VWF by targeting the neo-epitope Tyr1605, which only presents after ADAMTS13-mediated cleavage of VWF. Mature VWF is secreted from activated endothelium or circulates as a multimeric globular protein and unfolds under increased hydrodynamic shear forces exposing the ADAMTS13-cleavage site Tyr1605-Met1606 within the A2 domain of VWF [8,29,30]. Recently, a direct ELISA assay based on a commercially available monoclonal antibody targeting the same epitope as our VWF-A assay has been described, but so far quantification of ADAMTS13 proteolyzed-form of VWF is not commercially available [12]. This assay was used to evaluate the ratio between proteolysis and total VWF (VWF:Ag) focusing on VWD [12].

## VWF processing profile in Crohn's disease

Endothelial dysfunction is considered one of the etiological factors of IBD. It has been debated whether increased VWF in active IBD reflects damage of the endothelia or whether it is a response to acute inflammation caused by other mediators [31,32]. Besides the hemostatic roles of VWF, *in vitro* VWF directly binds and immobilizes extracellular DNA released from leukocytes, suggesting a role of VWF as adhesion molecule in leukocyte extravasation and inflammation by promoting leukocyte rolling and adhesion to endothelial cells which are hallmarks of inflammation [33]. Blocking antibodies against adhesion receptors such as integrin is a therapeutic target in IBD to avoid trafficking of leukocytes to the gut [34]. In addition, ADAMTS13 proteolyzed-form of VWF deactivates the complement system by promoting the generation of inactive C3b (iC3b). The complement system plays a key role in inflammation and the innate defense against pathogens, in which C3b is central to the function of all three complement pathways [35-37]. ULVWF do not affect the complement system, supporting the importance of VWF and its protease ADAMTS13 in inflammation and their potential as disease modifiers in IBD. We found significantly increased serum levels of both VWF-N and VWF-A in CD patients indicating increased deposition and ADAMTS13 proteolyzed-form of VWF compared to healthy subjects. Similarly to our findings with VWF-N, several other studies have found elevated plasma [38-40] and serum VWF levels [31,32,41] with worsening colitis disease activity [39]. Notable, the significant difference between healthy subjects and CD was much more pronounced for VWF-N than for VWF-A. There are indications which show significantly

decreased levels of ADAMTS13 in patients with chronic inflammatory disease [42] suggesting that ADAMTS13 might be directly associated with inflammation [43]. Also, ADAMTS13 might even serve as an anti-inflammatory mediator, as mice deficient in ADAMTS13 have a proinflammatory and prothrombotic response [43]. Increased VWF with accumulation at inflamed colonic submucosal vessels and a worsened DSS-induced colitis is found in ADAMTS13 deficient mice compared to wild-type mice [44]. One could speculate, whether an evaluated release of VWF due to endothelial damage and a decrease in levels of ADAMTS13 antigen and activity resulting in a diminished ADAMTS-13 mediated cleavage of VWF are pathological features in CD. As with CD, we found significantly high levels of VWF-N and VWF-A in patients diagnosed with non-IBD gastrointestinal diseases. Most of these patients had irritable bowel syndrome (IBS) and were suspected to have CD prior to their IBS diagnoses. This explains the unusual high-grade inflammation, measured as C-reactive protein (CRP)-levels > 5 mg/L, which is normally not a feature of IBS (Table 1). In IBD, studies have indicated that both plasma levels of ADAMTS13 antigen and activity were decreased in patients with high inflammation (high CRP levels), although the ratio between activity and total antigen remained normal [45]. Our study indicates that VWF is up-regulated in both CD and non-IBD patients, and we see that there is still enough ADAMTS13 activity to give an increase in the ADAMTS13 proteolyzed-form of VWF. Our data suggested that increased levels of VWF may be attributed to inflammatory conditions associated with gastrointestinal disease but not as a pathological effect of CD per se.

We did not find any significant difference between our biomarkers and behavior phenotypes of CD (data not shown). The cohort is too small to properly reflect the described disease group, and by only including CD and not UC patients we cannot review on the general relevance in IBD. Others have found serum levels of VWF in patients with IBD to be related to systemic inflammation [41]. CRP is a recognized marker of acute inflammation and is used as a marker for IBD [3,46], although CRP cannot differentiate CD from UC or verify an IBD diagnosis [46,47]. We did not find any correlations between our biomarkers and disease activity or CRP levels in CD patients. Similarly, we did not find any correlation between disease activity and CRP levels for this cohort. Increased circulating VWF serum levels have been shown to correlate with disease activity in UC, and in CD patients irrespective of disease activity [31]. Correlations between IBD disease activity, CRP and VWF levels have been debated in different studies [31,32,38,41,48]. Several other studies in IBD have found evaluated plasma [38-40] and serum VWF levels [31,32,41]. There may be some hesitation regarding measurements of VWF levels in serum, as VWF is a blood clotting protein that likely will be affected by blood preparation methods. However, we tested the level of released VWF-A in three kinds of plasma (EDTA, Heparin, Citrate) and serum from same donors and found with linear regression a strong correlation between these matrices ( $p < 0.0001$ ) (data not shown).

We wanted to examine protein turnover of VWF, which is the balance between protein synthesis and protein degradation. Our data suggest a normal turnover of VWF in these patients, as we did not find any significant discrepancies in the ratio of VWF-N/VWF-A among control subjects, CD patients and non-IBD patients. Based on this ratio, we cannot conclude with certainty that the multimeric structure of VWF was standard in CD patients before examining the change in VWF multimer distribution using electrophoretic multimer analysis. Also, the level of VWF and proteolysis of VWF multimers is modulated by age and ABO blood type [49]. We do not have access to any data on the blood types of the participants in this study.

## Conclusion

In conclusion, we report that CD patients had both increased formation but most importantly increased ADAMTS13 proteolyzed-form of VWF indicating a sustained elevated primary wound healing response as compared to healthy subjects. The use of serological biomarkers VWF-A and VWF-N as inflammatory and endothelium markers of intestinal disease should be investigated further, as these biomarkers could serve as supplementary non-invasive markers of intestinal healing in clinical practice and clinical trials.

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