Ultrasonographic evaluation of disease activity in Crohn's Disease

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“All disease begins in the gut”
Hippocrates (460-377 BC)

“What gets measured, gets managed”
Peter Drucker (1909-2005)

“Seek and you shall find”
Jesus (4 BC-30 AD), Matthew 7:7, The Bible

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Abbreviations

AIF	Arterial input function
ALARA	As low as reasonably achievable
B-mode	Brightness mode
BWT	Bowel wall thickness
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CDEIS	Crohn's Disease Endoscopic Index of Severity
CEUS	Contrast-enhanced ultrasound
CRP	C-reactive protein
CT	Computer tomography
DCE-US	Dynamic contrast-enhanced ultrasound
DICOM	Digital Imaging and Communications in Medicine
GI	Gastrointestinal
GIUS	Gastrointestinal ultrasound
HBI	Harvey Bradshaw index
IBD	Inflammatory bowel disease
IBS	Irritable Bowel Syndrome
ICC	Intra-class correlation
MHz	Megahertz
MI	Mechanical index
MRI	Magnetic resonance imaging
POCUS	Point-of-care ultrasonography
SES-CD	Simple Endoscopic Score for Crohn's disease
SUS-CD	Simple Ultrasound Score for Crohn's disease
TI	Thermal index
UC	Ulcerative colitis
UCA	Ultrasound contrast agents
US	Ultrasound

Scientific environment

This research was performed in the Bergen Research Group for UltraSound in Gastroenterology (BRUSE) at Department of Clinical Medicine, University of Bergen, and at National Centre for Ultrasound in Gastroenterology (NCUG), a National Advisory Unit located at Department of Medicine, Haukeland University Hospital.

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Abstract

Background: Crohn's disease (CD) is a chronic inflammatory disorder in the gastrointestinal (GI) tract, characterized by alternating periods of remission and relapse. Patients' symptoms do not reliably represent inflammatory activity and management should be based on objective evaluation. Currently, ileocolonoscopy is the established reference standard method for both diagnosis and monitoring disease activity in most cases, but holds several limitations restricting repeated use. Consequently, there is a need for safe, objective and accurate methods to measure the degree of inflammation and treatment response. Gastrointestinal ultrasound (GIUS) is a promising modality in assessing disease activity and may be a useful tool for aiding physicians improving treatment decisions.

Aims: The primary objective of the PhD project was to examine the usefulness of ultrasound in evaluating disease activity in patients with Crohn's disease. Specifically, we aimed to investigate the ability of Dynamic Contrast-Enhanced Ultrasound (DCE-US) to provide information of treatment effects (**paper I)**, to assess the diagnostic accuracy of GIUS in separating endoscopic remission from active disease (**paper II**), and to construct and validate a simple ultrasonographic activity index to quantify disease activity **(paper III)**.

Material and Methods: In **paper I,** 14 CD patients receiving medical therapy due to an acute exacerbation were examined with conventional- and contrast-enhanced ultrasound at four time points. In **paper II**, 145 CD patients scheduled for ileocolonoscopy were prospectively examined with GIUS within 2 weeks prior to or after the endoscopic procedure. The Simple Endoscopic Score for Crohn's disease (SES-CD) was used as a reference standard. In **paper III**, 164 patients scheduled for ileocolonoscopy were prospectively examined with GIUS, identically performed as in paper II. 40- and 124 CD patients were included in the construction- and validation cohorts, respectively.

Results: In **paper I**, we found significant differences in relative perfusion between responders and non-responders one month after treatment start. As a secondary

finding, differences in bowel wall layers were revealed, where the proper muscle- and submucosal layers were significantly thicker in non-responders at one and three months after treatment initiation, respectively. In **paper II**, we found that bowel wall thickness measurements on GIUS had 92.2% sensitivity, 86% specificity and 90.3% accuracy in separating the disease status. By adding color Doppler in sections with increased wall thickness and fecal calprotectin in sonographic colitis, the diagnostic accuracy improved. In **paper III**, we developed a simplified ultrasound score consisting of bowel wall thickness and color Doppler. The ultrasound score correlated well with SES-CD in both patient cohorts (Development cohort: $r=0.83$, $p<0.001$, Validation cohort: $r=0.78$, $p<0.001$), and revealed excellent interobserver agreement (Development cohort: ICC=0.95.Validation cohort: ICC=0.90).

Conclusions: We conclude that ultrasound is able to differentiate between patients with endoscopic remission and active disease, and a simple ultrasonographic scoring system is useful to evaluate the degree of endoscopic disease activity in CD. Furthermore, GIUS enables prediction of treatment effect shortly after treatment start, thus improving treatment decisions.

List of publications

The PhD dissertation is based on the following papers, referred to in the text by their roman numerals:

I. Saevik F, Nylund K, Hausken T, Odegaard S, Gilja OH. Bowel perfusion measured with dynamic contrast-enhanced ultrasound predicts treatment outcome in patients with Crohn's disease.
Inflammatory bowel diseases. 2014;20(11):2029-37.

II. Saevik F, Gilja OH, Nylund K. Gastrointestinal ultrasound can predict endoscopic activity in Crohn’s disease
Accepted in Ultraschall in Med, Apr 24 2020.

III. Saevik F, Eriksen R, Eide GE, Gilja OH, Nylund K. Development and validation of a simple ultrasound activity score for Crohn’s disease.
Accepted in Journal of Crohn’s and colitis, June 6 2020.

1. Introduction

1.1 Crohn's disease

1.1.1 Background

Inflammatory bowel disease (IBD) comprises two major disorders, ulcerative colitis (UC) and Crohn's disease (CD) (1). All studies included in the thesis were performed on CD patients, mainly focusing on ultrasonographic characteristics and assessments of these patients.

Crohn's disease is a chronic inflammatory disorder affecting the gastrointestinal (GI) tract, characterized by an alternating course between remission and relapse. Transmural inflammation, skip lesion distribution, and several severe complications are other distinctive features of CD (2). The disorder is usually diagnosed in young adults, and consequently, patients are affected for years (3). The disease course varies between sustained quiescence in some patients to severe inflammation leading to serious complications necessitating surgical treatment in others (4). Due to the unpredictable course, individual adjustments of treatment and follow-up is mandatory.

1.1.2 Epidemiology

During the last decades, the incidence and prevalence of CD have been increasing worldwide (5, 6). Still, significant geographical differences exist, with higher frequency in western, industrialized countries (5-7). Further, higher incidence rates are reported in northern compared with southern latitudes in Europe (8) and North America (9), as well as an East-West gradient within Europe (7). In Norway, the incidence and prevalence of CD are 22/100 000 and 258/100 000, respectively (10).

1.1.3 Etiology

Even though CD has distinctive pathologic and clinical traits, the pathogenesis remains poorly understood. Currently, the main hypothesis suggests that environmental factors trigger epithelial dysfunction on genetically susceptible individuals leading to an inappropriate immune response against the microbial flora, causing inflammation and tissue damage (2, 11, 12). To date, no single immune-triggering environmental factor is identified, however, industrialization and adoption of western lifestyle are related to the increasing incidence of CD in developing countries (13). Smoking is the best documented risk factor associated with CD, but cannot explain the increased global incidence, suggesting multifactorial etiological triggers (13).

1.1.4 Disease manifestation

Patients are phenotypically categorized according to the Montreal classification (14), where age at diagnosis (<16 years (A1), 16-40 years (A2), and >40 years (A3)), location (ileal (L1), colonic (L2), ileocolonic (L3), and isolated upper disease (L4)), and behavior (inflammatory (B1), stricturing (B2), and penetrating (B3)) are characterized. Additionally, a perianal modifier (p) may be added when present (Figure 1). Where ulcerative colitis affects the colon in a continuous pattern, CD may affect the entire GI tract from the mouth to the anus in a skip pattern. Still, the majority of CD lesions are usually limited to the ileum and colon, where approximately 30% of CD patients present with ileal (L1), colonic (L2), or ileocolonic (L3) affection (3, 15). In contrast, approximately 5% present with upper disease (4). The disease location seems to remain stable, while the behavior varies over time (4, 15, 16). Perianal involvement occurs in about 10-25% (15).

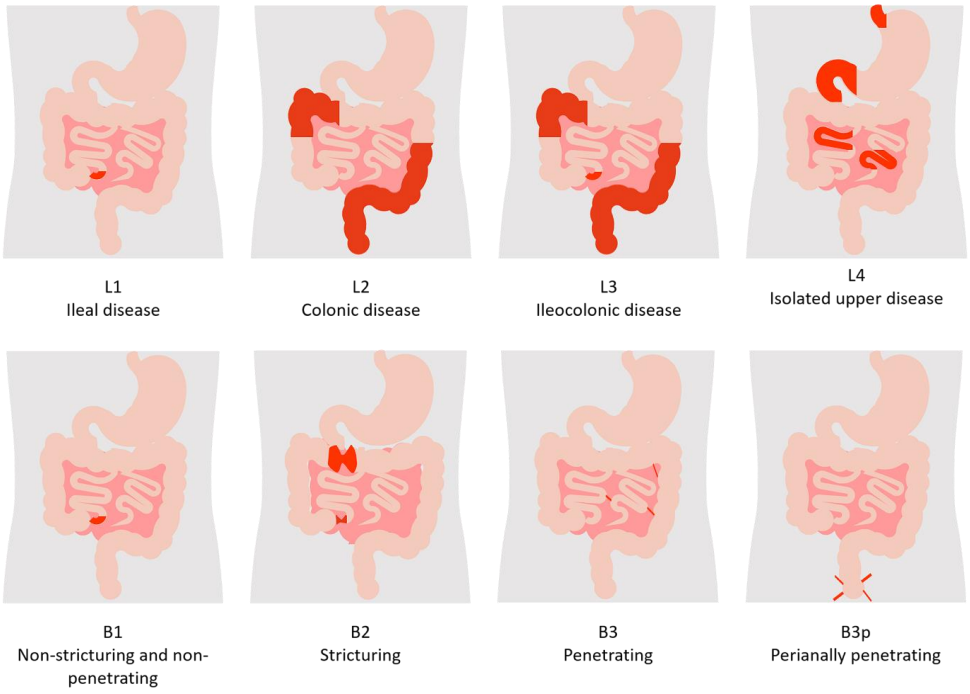

Figure 1: Location and behavior categorized according to the Montreal classification.

Clinical presentation varies due to differences in disease location, behavior, and activity. The intestinal inflammation may cause symptoms such as chronic diarrhea, possibly with blood or mucus, abdominal pain, and weight loss, as well as general symptoms such as fever, malaise, and fatigue due to systemic inflammation (2, 17). Most patients present with a non-stricturing/ non-penetrating behavior (B1) at the time of diagnosis (15, 16). As disease behavior changes over time, approximately half of the patients develop stricturing or penetrating complications (4, 18). Stricturing disease causing bowel obstruction could present as post-prandial abdominal pain, nausea, and vomiting (2). In penetrating disease, the development of fistulas or abscesses occur. The formation of a fistula enables penetration of luminal content throughout the intestinal wall into other bowel segments or organs, and the symptoms

depend on its location (2). Abscesses may present as fever and abdominal pain, and a tender abdominal mass may be palpated (19). Finally, extraintestinal manifestations affecting joints (arthritis and ankylosing spondylitis), skin (erythema nodosum and pyoderma gangrenosum), eye (uveitis) or liver (primary sclerosing cholangitis) (20) may further complicate the disorder.

1.1.5 Disease course

CD patients may be classified into four primary disease courses, suggesting that no single management plan will suit all patients and should thus be tailored to the individual's needs (4, 21). In a population-based study in Norway, the investigators found that 43% of patients may have a mild disease course and do not require long-term intensive therapy. Still, most patients are likely to alternate between remission and relapse (15, 21), and may benefit from early aggressive long-term management. Even though clinical activity may diminish over time in some patients, the rate of acute deterioration and development of complications is high during the first ten years after diagnosis, leading to a naturally progressive destructive disease course (4, 15). Requirements for corticosteroids, high C-reactive protein (CRP) at diagnosis, smoking, early onset, and perianal disease are factors that may predict a disabling course and poor prognosis (15, 22-25). Even though CD might be debilitating, the overall mortality is not increased (26).

Chronic transmural inflammation may cause excessive damage of the intestinal wall leading to fibrotic changes due to aberrant healing failing to restore normal tissue architecture (27, 28). Strictures occur in approximately half of all CD patients (27) and are subdivided into mainly fibrotic, inflammatory, or mixed types (29). Distinguishing between the various clinical expressions is important due to different treatment strategies (17, 27, 30), but remains challenging.

1.1.6 Morphology

Gross examination of resected specimens typically reveals bowel wall thickening, serosal fat wrapping (“creeping fat”), and longitudinal ulcers in a discontinuous pattern with a sharp demarcation to the adjacent uninvolved bowel (31). Severe inflammation could lead to the formation of penetrating complications such as fistulas, sinuses, or abscesses, which are mainly found in patients with ileal or ileocolonic involvement. Strictures are identified as stiff bowel segments with a narrowed lumen (31).

By microscopic examination, discontinuous chronic inflammation, crypt irregularity, and non-caseating granuloma are histological features allowing for diagnosing CD. Moreover, muscular- and neural hypertrophy, increased neutrophilic infiltration into the epithelial layer, and proximal affection are additional histological features. Currently, no single diagnostic feature is available; still, the presence of granuloma together with one additional finding could establish the diagnosis (31). Although considered as the hallmark of histological diagnosis in CD, the presence of non-caseating granulomas ranges from 20-60% of cases (32-34) and is more frequent in pediatric patients (32). Moreover, the formation of granuloma seems to be associated with aggressive phenotypes (33, 35) but may regress during treatment (34). Even though non-caseating granulomas are lacking in a substantial number of patients, the histological diagnosis could be established by other characteristic microscopic features (31).

Several immune-mediated factors promote increased angiogenesis of the bowel wall, perpetuating chronic inflammation (36, 37). Moreover, impaired local tissue perfusion due to microvascular dysfunction creates an ischemic environment which may further sustain the inflammatory state and facilitate neovascularization (37, 38). Enhanced angiogenic activity due to neovascularization can be reflected by increased microvessel density and expression of vascular- and pro-inflammatory mediators (39, 40). Furthermore, increased blood flow occurs in acute inflammation while it is

reduced in chronically inflamed segments (41). Hence, measurements and quantification of microvessel density and perfusion may aid evaluation of the degree of inflammation.

1.1.7 Diagnostic modalities and indices

Currently, no single gold standard method for CD exists. Both diagnosis and disease activity assessments are based on a combination of clinical, biochemical, radiological, endoscopic, and histological evaluations (42, 43).

1.1.7.1 Clinical assessment

Clinical assessment of CD patients is performed in both daily practice as well as in clinical studies. A structured medical history mapping clinical presentation, evolution of symptoms, risk factors, and general condition is commonly conducted. It is further accompanied by physical examination where cardiovascular status, calculation of body mass index, abdominal-, perianal- and digital-rectal examinations are performed (44). Clinical findings include identification of tender masses, palpable resistance, and abdominal pain by palpation, as well as fissures, fistulas, or abscesses during careful examination of the perianal region (17). Several scoring systems for measuring clinical disease activity are available, including the Crohn's disease activity index (CDAI) (45) and the simpler Harvey-Bradshaw index (HBI) (46). The CDAI consists of a seven-day evaluation of eight clinical and laboratory variables. All variables are weighted, and finally, a total score is calculated. CDAI <150 is regarded as clinical remission, while 150-219 as mild, 220-450 as moderate, and >450 as severe disease activity (47). HBI is a simplified derivate of the CDAI, consisting of five clinical parameters. A HBI score of <5 represents clinical remission, while 5-7 is regarded as mild, 8-16 as moderate, and >16 as severe disease activity (47). The concordance between the indices is well-defined, where a change of 3 points in the HBI corresponds to a 100-points change using the CDAI (48).

Improvements of clinical activity measurements are commonly used as endpoints in clinical trials, still, they do not sufficiently represent reliable measures of inflammatory activity (49, 50), and should be supplemented by objective markers.

1.1.7.2 Biochemical evaluation

Biochemical markers are used routinely in daily clinical practice for initial diagnosis as well as in follow-up examinations of IBD-patients (17), but specific tests are currently lacking. Common, but unspecific findings include general inflammatory markers such as elevated levels of CRP, erythrocyte sedimentation rate, leukocytes and thrombocytes (51), as well as low values of albumin (51) and hemoglobin due to chronic inflammatory activity or iron/vitamin deficiency (52).

Stool samples can be analyzed for fecal biomarkers; Calprotectin is a neutrophil protein reflecting the migration of neutrophil leucocytes in the gut, representing a surrogate marker of bowel inflammation (53). Fecal calprotectin has higher diagnostic accuracy than CRP (54), correlates well with endoscopy (55), and is useful for disease activity monitoring (55, 56). Further, the biomarker is an accurate screening tool for IBD (57, 58), including exclusion of irritable bowel disease (IBS) (59). However, it seems better suited for evaluating activity in UC than CD (54), a lower accuracy is achieved in small-bowel compared to colonic CD (60), and it can be elevated in other inflammatory conditions as well as in neoplasia (61, 62).

1.1.7.3 Endoscopy

Ileocolonoscopy is considered the reference standard method for both diagnosis and evaluation of disease activity in CD located in the colon and terminal ileum. It enables an excellent assessment of the mucosal surface and permits biopsy sampling

for histological evaluation. The presence of discontinuous lesions of aphthous, deep, stellate, linear, or serpiginous ulcers, stenosis, fistula, and cobblestoning of mucosa are typical endoscopic features of CD. Additionally, affection of the terminal ileum and perianal involvement further support the CD diagnosis (63). For activity monitoring and as an outcome measure in clinical trials, endoscopic activity indices are recommended (64). Crohn's Disease Endoscopic Index of Severity (CDEIS) (65) was previously the only validated endoscopic activity score. However, the score is cumbersome and time-consuming, making the method unsuited for daily clinical practice. Consequently, the Simple Endoscopic Score for Crohn's Disease (SES-CD) was developed (66). SES-CD correlates well with CDEIS (67) and may replace CDEIS in clinical trials as well as in routine work. Both scoring systems describe the rectum, left colon (descending colon and sigmoid), transverse colon, ascending colon, and the terminal ileum. The parameters included in the CDEIS-score are the presence of deep ulcers, superficial ulcers, surface involved by disease, ulcerated surface, ulcerated stenosis, and non-ulcerated stenosis. By using the SES-CD the size of ulcers, ulcerated surface, affected surface, and presence of stenosis are evaluated using a quantitative score of 0-3 per parameter per segment. The Rutgeerts score (68) is a scoring system developed for evaluation of post-operative recurrence of CD. Despite being recommended in international guidelines (69), no formal validation has been performed (63). Mucosal healing is absence of inflammation at endoscopy and has emerged as an important therapeutic goal in IBD (70). Even though consensus of endoscopic response and remission are recently established (71), there is currently no formally validated definition of mucosal healing (69). Common definitions of mucosal healing include SES-CD 0-2, CDEIS 0-3 (71), absence of mucosal ulcerations, or CDEIS/SES-CD = 0 (63). Despite numerous advantages of using the endoscopic quantitative indices to rate the severity of inflammatory activity, the complexity of the scoring systems limits their use in clinical practice (43).

There are some major limitations using endoscopy. First, there is no knowledge whether inflammation persists in deeper layers of the bowel wall. Furthermore, most of the small bowel and peri-intestinal complications cannot be visualized (72).

Finally, the examination is invasive causing considerable patient discomfort (73) making it less suited for repeated examinations.

1.1.7.4 Imaging

Due to the above-mentioned limitations of endoscopy, cross-sectional imaging modalities such as Ultrasound (US), Magnetic Resonance Imaging (MRI), and Computed Tomography (CT) are needed to provide complementary information. These imaging modalities have high and comparable diagnostic accuracies for initial assessment, follow-up examinations, and complications of the disease (42, 74-76).

CT enterography is a fast and widely available imaging modality enabling detection of CD, as well as evaluation of disease activity, extent, and complications with high accuracy (77). Unfortunately, ionizing radiation exposure of CT represents a major disadvantage of this imaging modality. As repeated examinations increase the risk of cancer (78, 79) its use should be limited, particularly in young patients. Although ionizing hazards may be reduced using low-radiation-dose CT protocols (80, 81), non-ionizing imaging modalities are preferable in most clinical settings (42).

MRI is a non-radiating imaging modality providing excellent visualization of a wide range of pathological features of CD, ideal for small bowel evaluation (42, 82-84). Due to the lack of ionizing radiation, the technique is well-suited for follow-up examinations and disease monitoring (85). Accordingly, MRI is the current standard for small-bowel assessment in CD (17). Disease activity may be quantified using dedicated scoring systems, where the MaRIA score may be most suitable (86). This validated activity index corresponds well with endoscopy to evaluate disease activity, severity (87, 88), and ulcer healing (89). Further, a simplified derivate of the activity index was recently developed (90), which may reduce the need for repeated ileocolonoscopies in activity monitoring. Although advocated as the preferred cross-

sectional imaging modality, MRI is expensive, resource intensive, relatively inaccessible, and contraindicated in some patients (91).

1.2 Ultrasound

1.2.1 General

Ultrasound (US) is high-frequency sound waves exceeding the upper audible limit in humans, capable of constructing greyscale images in real-time. Frequencies between 2-15 megahertz (MHz) are commonly used in abdominal diagnostic imaging, and in contrast to X-ray modalities utilizing attenuation, ultrasonography is based on reflection of sound waves (92).

Piezoelectric crystals inside the ultrasound transducer generate acoustic waves by transforming electrical voltage. The ultrasound waves are emitted inside the body and when reaching tissue boundaries, parts of the sound waves are reflected towards the probe, enabling conversion of ultrasound waves to electrical voltage, finally creating a grey-scale image (93).

Sound waves are characterized by the frequency (f), wavelength (λ), and speed of the sound (c). The frequency is defined as the number of oscillations per second and has the unit of Hertz (1 Hertz = 1 cycle per second). The wavelength is referred to as the distance between two identical, consecutive coordinates on the waveform and has the unit of millimeters (mm). The speed of the sound has units of meter/second (m/s) and is determined by tissue characteristics ranging from 331 m/s in air to 3500 m/s in bone (93). However, an average of 1540 m/s is commonly used as the differences between most soft tissues are negligible (93). The relationship between frequency, wavelength, and speed is given by the formula:

$$\lambda = c/f \qquad (1)$$

Since the propagation speed is treated as constant, optimization of the image is determined by the inversely related wavelength and frequency parameters. By

increasing the frequency, a higher resolution of the US image at the expense of depth penetration is obtained. Conversely, lower frequencies increasing the wavelength are chosen for appropriate visualization in structures that are deeply located.

1.2.2 B-mode

In Brightness mode (B-mode) imaging, the reflected US waves are displayed as dots of varying brightness proportional to the amplitude of the return echo, positioned according to the corresponding depth of the interface reflector. The final B-mode image is a combination of all returned echoes registered along the scan lines of multiple piezoelectric crystals inside the US transducer (92).

1.2.3 Doppler

Doppler ultrasound enables evaluation of the circulation by utilizing the Doppler Effect, defined as a change in frequency between the reflected- and emitted US wave, due to relative motion between the observer and the reflector (93). The Doppler effect (Δf) created by moving erythrocytes enables blood flow velocity (v) measurements, as expressed by the equation:

$$\mathrm{v} = \frac{\mathrm{C} \cdot \Delta f}{2f0 \cdot \cos\theta} \qquad (2)$$

Further, knowledge of the ultrasound speed (C), frequency of the transmitted US (*f0*), and the cosine of the angle between the US beam and direction of blood flow ($\cos\theta$) are needed for performing the calculation.

Color Doppler sonography enables evaluation of vessel patency as well as blood flow directions, where flow towards and away from the probe usually are coded as red and blue, respectively. Further, increased color intensity correlates with elevated flow

velocity, while a mosaic of colors may be seen in turbulent flow (94). Power Doppler sonography merges each frequency shift in the sampling volume, thus lacking the ability of flow direction assessments. The amplitude of reflected signals correlates with the number of erythrocytes regardless of velocity, hence, the sensitivity of small vessel detection increases (94).

1.2.4 Contrast-enhanced ultrasound

Doppler ultrasound is a well-established method to detect high-velocity blood flow; however, the availability for evaluating perfusion in organ parenchyma is limited due to lower velocities, making it difficult to discriminate blood flow from tissue motion. Contrast-enhanced ultrasound (CEUS) combines conventional ultrasound with ultrasound contrast agents (UCA), enabling evaluation of the microvasculature (95-97). The role of CEUS is well-established in liver imaging (98), and several new applications have emerged during the last decades (99, 100).

1.2.4.1 Ultrasound contrast agents

Ultrasound contrast agents (UCA) are microbubbles consisting of a gas-filled core encapsulated by a biocompatible shell. The microbubbles are made small enough to pass capillary beds and no extravasation of contrast agents occurs, thus acting as a true intravascular tracer (101). Renal monitoring is usually unnecessary as the UCA is eliminated through the lungs (102, 103).

Commercially available UCAs containing coated air bubbles were introduced in the nineteen nineties (104). Currently, second-generation agents containing biologically inert high molecular weight gases are chosen due to improved backscattering abilities as well as prolonged lifetime. In Europe, Sonovue (Bracco, Milan, Italy), a sulfur hexafluoride filled microbubble, is the most commonly used UCA.

Due to high compressibility of the UCA the bubbles contract and expand in response to the application of low energy ultrasound with appropriate resonance frequency, while in contrast, the surrounding tissue is relatively incompressible. Also, distorted non-linear reflections are produced, which can be differentiated from tissue-derived signals. The oscillation frequency of the microbubbles is inversely related to its size and the frequency of the US beam (101, 105), hence, higher doses of contrast agents are required to compensate size-frequency dissonance (106). When exposing the UCA to higher intensities, the coating shell disrupts due to rarefaction forces (high MI), releasing the encapsulated gas (101, 107).

Various techniques have been developed to discriminate between non-linear UCA reflections and tissue-derived signals. In the pulse-inversion technique, two pulses of US with inverted phases are transmitted. The tissue-derived inverted linear echoes are summed thus canceling each other out, while in contrast, non-linear reflections from the microbubbles amplify each other when summed (101, 107, 108). In amplitude modulation, two US pulses with altered amplitudes are transmitted. Linear echoes are canceled out by subtracting the reflected pulses, while non-linear UCA-derived signals at various frequencies remain (107, 109). Modern US scanners most commonly combine phase inversion and amplitude modulation.

1.2.4.2 Perfusion estimates

Contrast-enhanced ultrasound (CEUS) enables quantification of the microvasculature (110, 111) due to a directly proportional relationship between the backscattered signal intensity and the microbubble concentration (112). Further, the analysis of contrast enhancement over time, dynamic contrast-enhanced ultrasound (DCE-US), enables estimation of perfusion (95, 113). The UCA remains intravascular, which is necessary for accurate perfusion measurements (95). There are currently two established administration methods, Bolus tracking and Burst replenishment (95, 114), yielding

different measurements. The preferred method depends on study purpose or clinical experience. Currently, the Bolus tracking technique is used more frequently than Burst replenishment in non-cardiac applications (95).

In Bolus tracking, the contrast agents are administered as a bolus injection through a peripheral vein and after passing through the pulmonary circulation, the microbubbles reach the systemic circulation and the region of interest in the arterial phase. Finally, the contrast intensity gradually diminishes in the venous phase. Time-intensity data is detected during the arterial phase, where the arrival of UCA and decline in contrast enhancement is registered. By using appropriate software, the time-intensity data is fitted to a standardized curve from which several relative perfusion parameters are derived (95, 113).

Perfusion quantification analyses are commonly performed on log-compressed versions of the actual ultrasound intensities. However, the only mathematically valid method is by using linear echo power data (115), which is proportional to the bubble concentration. Due to difficulties in obtaining linear raw data, re-linearizing conversion algorithms are available in modern quantification software applications (113), which is an acceptable alternative if the gain is properly adjusted and the dynamic range of log-compression exceeds 45 decibel (116).

1.2.5 Safety

Ultrasound is considered a safe modality with no proven harmful effects. Still, precautions should be made as sustained exposure with high power output produces heating and pressure changes in tissues which may be potentially hazardous (117). Heating occurs when energy from a propagating ultrasound wave is absorbed and converted to heat. An elevated temperature may affect normal cell functions, still, evidence of clinically significant deleterious effects is lacking (117). For guidance, an estimate of the temperature rise displayed as a thermal index (TI) is provided (93).

$$TI=W/W_{deg} \qquad (3)$$

W=the power exposing the tissue, W_{deg}=the power necessary to raise the temperature by 1°C. In presence of bones, an increased heating effect occurs due to higher absorption of US waves, thus, three versions of TI may be provided: soft tissue (TIS), bone at focus (TIB), and cranial (bone at surface) (TIC) (93).

Pressure changes caused by compression and rarefaction of propagating ultrasound waves may lead to mechanical disturbances in tissues. High acoustic pressures could potentially induce unfavorable inertial cavitation, thus, a mechanical index (MI) estimating the likelihood of such induction is provided (93).

$$MI=P_r/\sqrt{f} \qquad (4)$$

P_r= the peak rarefaction pressure, f= ultrasound frequency.

Owing to the potentially deleterious effects of ultrasound, the thermal and mechanical indices are mandatorily displayed on ultrasound scanners. The *As Low As Reasonably Achievable* (ALARA)-principle is generally recommended, where TI >6 in adults and >3 in obstetrics, as well as MI >1.9, should be avoided (118, 119).

Sonovue has a good safety profile with few and mild side effects, however, rare cases of serious adverse reactions have been reported in the literature, and emergency precautions should be taken (120-124).

1.2.6 Ultrasonographic features of Crohn's disease

Ultrasound is non-invasive, cost-effective, well-tolerated by patients and can be performed bedside, making it well suited for repeated examinations. The impact of gastrointestinal ultrasound (GIUS) has increased significantly during the last decades, and dedicated GIUS-guidelines have recently been provided (125, 126). Due to logistic, safety, and economic reasons, ileocolonoscopy, CT, and MRI cannot be performed on a regular basis, and consequently, GIUS might be a useful supplement

in IBD management. A recent appraisal of the literature revealed good accuracy of US in diagnosis and mapping of complications, while poor and limited evidence was found for disease activity assessments (127). Previous meta-analyses show that there is a good correspondence between US, CT, and MRI in detection of the disease and complications as well as evaluating the extent and severity of the disease (74, 75). However, in light of recent technological advances of all modalities, updated meta-analyses are warranted.

Both low- and high-frequency probes are required to perform a thorough GIUS examination. A low-frequency curvilinear transducer provides good depth penetration, ideal for overview, identification of gross pathology as well as the examination of the deeply located rectum. A detailed examination of the distal ileum and colon is performed by systematic scanning from the terminal ileum and further distally, using high-frequency linear probes. Most of the small bowel is difficult to track due to a tortuous course, thus, a systematic four-quadrant examination is recommended. Then, the dorsal abdominal wall should be identified to ensure that all bowel segments are included in the scanning area. No preparation of the patient is required, but it is recommended that patients fast for at least four hours to reduce intestinal gas (125).

Normally, the intestinal wall is less than two mm measured by ultrasound (128), and by using high-frequency probes, five to nine wall layers can be delineated depending on the transducer frequency. There are clear correlations between the wall layers revealed by ultrasound and histology (Figure 2), even though slight differences occur (125, 129).

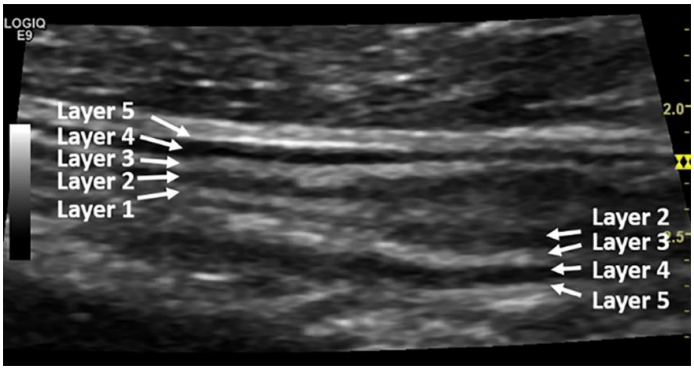

Figure 2: B-mode image of the author's healthy bowel. Five demarcated layers correspond partly to the intestinal wall layers. In practice, the hypoechoic layer 2 corresponds to the mucosa, whereas the hyperechoic layer 3 correlates with the submucosa, the hypoechoic layer 4 to the proper muscle, and the hyperechoic layer 5 to the serosa. The hyperechoic layer 1 corresponds to the interface between the mucosa and intestinal lumen.

Increased bowel wall thickness (BWT) is the most common and important parameter for detecting CD (42, 126) (Figure 3), yielding high sensitivity and specificity (130, 131). A recent meta-analysis showed that wall thicknesses exceeding three mm yield sensitivity and specificity of 89% and 96%, respectively. By increasing the threshold to four mm or higher, a sensitivity of 87% and a specificity of 98% were found. (131). Additionally, BWT may be useful in evaluating disease activity (132, 133), detection of postoperative recurrence (134) and prediction of surgery (135). Until recently (125) international guidelines regarding BWT measurements were lacking. Standardization of BWT assessment appears a prerequisite for high interobserver agreement (136), as inadequate instruction has resulted in poorer agreement rates even when performed by expert sonographers (137).

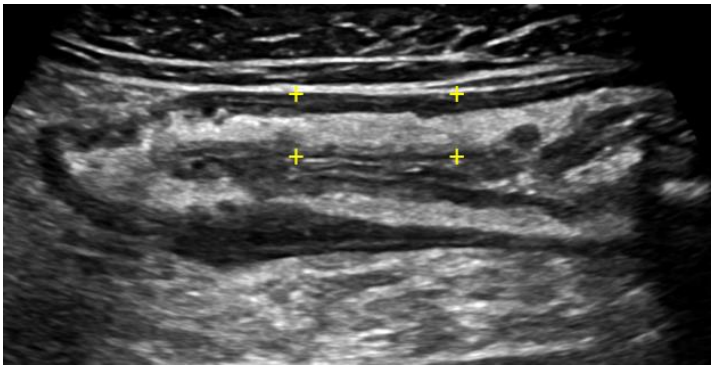

Figure 3: A thickened bowel wall (demarcated by yellow calipers) with normal stratification in a patient with Crohn's disease.

Echo patterns may differ in CD, ranging from preserved stratification and thickened submucosal layer (138, 139) to abrogation of the wall layers. Focal loss of bowel wall stratification is associated with ulcerations (140) (Figure 4), while diffuse disruption may be caused by severe transmural inflammation (126, 141), increasing the risk of surgery (142). In contrast, intact stratification and thickening of the proper muscle layer are indicative of fibrosis (139, 141). Moreover, a fibrofatty proliferation of the surrounding mesenteric fat is commonly present in patients with disease activity (143). It typically presents as echo-rich tissue encircling the affected bowel segments (Figure 4), however, a hypoechoic appearance may be seen in long-standing disease (144). Other common, but unspecific extraintestinal findings include enlarged mesenteric lymph nodes and free fluid (126).

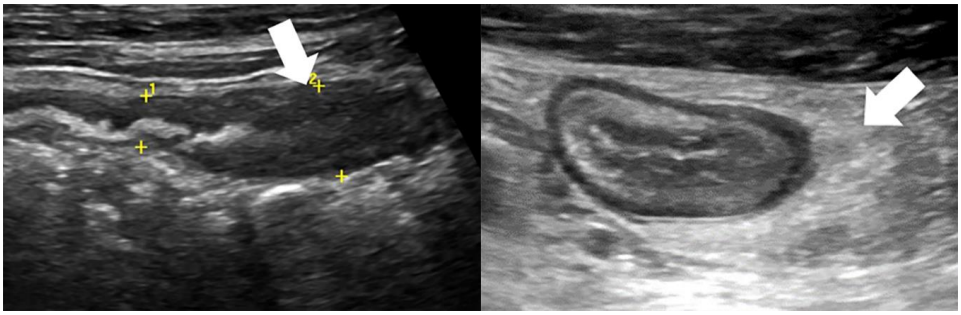

Figure 4: The left panel shows a focal loss of stratification (arrow), while the image in the right panel displays inflammation in the mesenteric fat (fatty wrapping) (arrow).

Several complications of CD can be detected by US. Intestinal stenosis appears as a segmental increase in bowel wall thickness with a narrowed lumen and prestenotic dilatation exceeding 2.5 cm (Figure 5), often accompanied by hyperperistalsis. Fistulas are visualized as hypoechoic tracts between intestinal loops and other tissues. In addition, content of air bubbles seen as hyperechogenic structures within the duct may be present. Abscesses usually appear as irregular hypoechoic peri-intestinal structures, containing echo-rich air bubbles (126, 145).

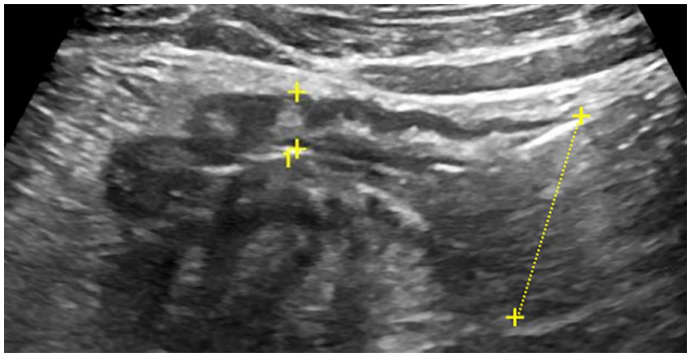

Figure 5: Stenosis in the terminal ileum with prestenotic dilatation.

In active CD, increased angiogenesis featuring neovascularization and local dysregulation of the blood supply is present (38, 39), allowing for quantification of the microvasculature as a means of activity assessments. Color Doppler measurements of the GI wall enables differentiation between active and inactive disease and correlates with endoscopy, histology, and CDAI (146-151) (Figure 6), and there is a negative correlation between Doppler signals and fibrosis (152). Furthermore, color Doppler measurements may aid physicians to monitor disease status and evaluate treatment effect (132, 133). However, color Doppler has limited resolution for small vessels with low-velocity blood flow (153), possibly making the modality less sensitive for microvascular changes.

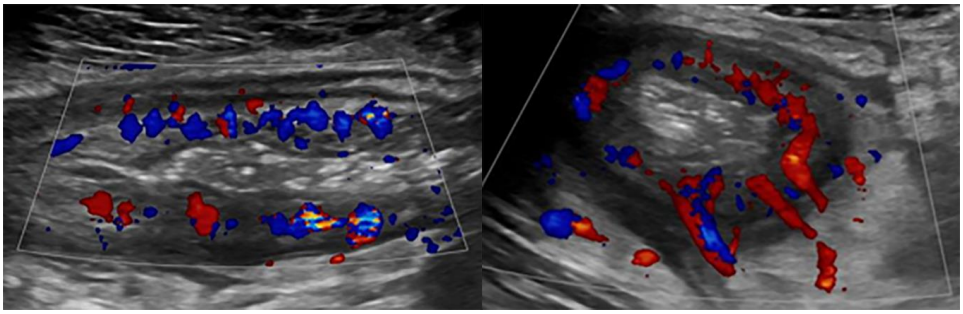

Figure 6: A bowel loop with increased color Doppler signals is depicted in longitudinal- and transverse sections in the left and right panel, respectively.

Previous meta-analyses revealed that CEUS is well-suited for detection of active CD with high sensitivity and specificity (154, 155), although affected by significant heterogeneity between the included studies. Disease activity evaluation may be performed using semi- (156, 157) or quantitative methods (151, 158-163), where increased contrast enhancement corresponds to inflammation. Further studies suggest that CEUS might be useful in differentiating between inflammatory and fibrotic lesions, where low values of relative signal intensities (152, 164-166) and absolute perfusion parameters indicate fibrosis (139). In addition, early evaluation of CEUS-derived perfusion parameters may be useful in determining treatment outcome, thus

enabling improvements of management (167-169). The main application of CEUS in clinic, however, is to differentiate between abscesses and phlegmons (Figure 7) (99, 170).

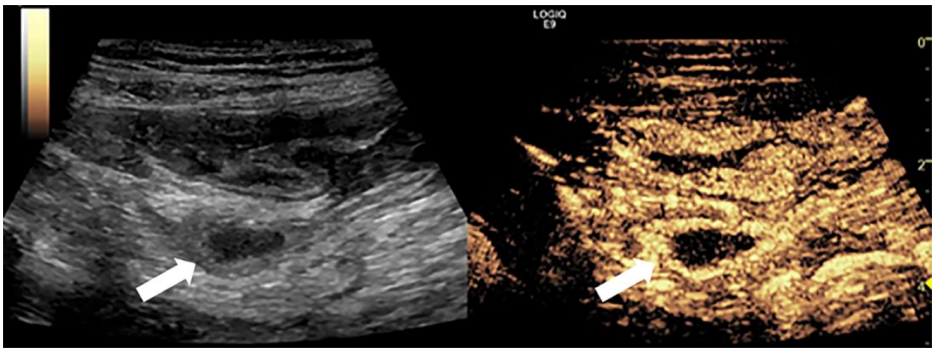

Figure 7: Ultrasound images of an abscess. The B-mode image in the left panel displays a hypoechoic lesion (arrow). The corresponding contrast image in the right panel shows a hypoechoic central structure with contrast enhancement in the peripheral zone.

In the treat-to-target era, reaching objective endpoints are favorable as beneficial changes in the disease course are provided. Mucosal healing has emerged as the main therapeutic target, but endoscopy fails to detect persistent inflammation in deeper layers in transmurally affected bowels. Hence, transmural healing defined as normalization of the bowel wall at cross-sectional imaging may be a more appropriate treatment goal (171). Studies report that sonographically measured transmural healing correlates well with mucosal healing but seems harder to achieve (163, 172-174) and may represent a more profound level of healing. In a recently published retrospective study, transmural healing measured by ileocolonoscopy and MRI-enterography was found to be superior in any outcome compared to mucosal healing alone (175). Still, the clinical role of transmural healing remains to be determined (171, 176).

In the hands of trained clinicians, GIUS substantially improves clinical decision making (177) and is useful for activity monitoring (132). Suitable scoring systems for measuring inflammatory activity are available for several diagnostic modalities (43).

However, the methodology for development is inadequate in most ultrasound indices (178).

1.3 Treatment of Crohn's disease

Inflammatory lesions of CD are treated medically with topical or systemic steroids, immune modulators, and biologics, while endoscopic dilatation or surgical resection is performed in fibrotic segments (17, 27, 30, 179). Appropriate management depends on accurate determination of disease activity, site, and behavior. The traditional approach of CD management is based on alleviation on patients' symptoms using a "step-up" strategy, starting with less potent medication and further escalation if inadequate effect (180). However, due to mismatch between symptoms and disease activity, persistent subclinical inflammation may go undetected, ultimately leading to irreversible bowel damage (4, 181). Thus, a new management paradigm has emerged treating beyond clinical symptoms to objective endpoints, where mucosal healing is considered as the main therapeutic target (182). Acquiring mucosal healing is correlated with less hospitalization, relapse rates, surgery, and bowel damage (70, 183-186), and may be key to change patient outcomes (187, 188). Further evidence suggests that a better optimization of therapy may be achieved when based on objective markers of inflammation rather than on symptoms alone (189). Topical or systemic steroids are recommended to induce remission in CD, with further escalation to immunosuppressants when necessary (17, 179). Still, a "top-down" strategy with early introduction of biologics may be appropriate in patients with severe disease or poor prognostic factors (179, 190). Unfortunately, some patients have suboptimal response to biologics or experience drug failure over time (191, 192). Furthermore, these drugs are expensive and have potentially serious side effects (193). Consequently, frequent follow-up examinations using simple, accurate tools for objective evaluation of disease status are needed for improved treatment management.

2. Rationale and aims

2.1 Rationale

Many CD patients suffer significantly due to impaired bowel function. Affected individuals are usually diagnosed of young age, thus numerous follow-up examinations are needed to evaluate disease activity and treatment effect. Due to a mismatch between patients' symptoms and the degree of disease activity, there is a need for objective measurements of the degree of inflammation in the intestinal wall in order to improve management. Gastrointestinal ultrasonography is potentially useful for evaluating changes in disease activity in affected bowel segments and may thus be a useful recourse in facilitating patient care.

2.2 Aims

The principal aim was to investigate the ability of ultrasonography to assess disease activity in patients with CD. The thesis is based on three papers, with each specific objective:

I. To evaluate whether DCE-US-derived perfusion parameters can be used to monitor disease activity and treatment effect in patients with CD. Secondly, we aimed to investigate the most appropriate time to perform the follow-up examinations.

II. To assess the diagnostic accuracy of GIUS in separating CD patients in endoscopic remission from patients with active disease.

III. To construct, validate, and assess interobserver agreement of a simple ultrasonographic scoring system for evaluation of disease activity in CD.

3. Materials and methods

3.1 Study population

In **paper I**, 14 CD patients (nine men) scheduled for treatment with either corticosteroids or biologics due to disease flare-up (defined as CDAI > 150 points) were prospectively recruited from the outpatient clinic or at the ward at the Section of Gastroenterology at Haukeland University Hospital, Bergen, Norway. All patients completed four follow-up examinations during 12 months.

In **paper II** and **III**, we prospectively included 145 (58 men) and 164 (66 men) patients, respectively, who were referred to ileocolonoscopy as part of standard care at the Department of Medicine at Haukeland University Hospital, Bergen, Norway, (Paper II and III) and the Department of Medicine at Ålesund Hospital, Norway (Paper III). All study participants in **paper II** were also included in **paper III**.

A detailed description regarding inclusion and exclusion criteria are provided in the associated papers.

3.2 Study design and enrolment

All studies were observational. The ileocolonoscopic examinations, decision-to-treat, or changes in medical therapy were performed as part of usual care.

Paper I was designed as a prospective follow-up study, examining patients at four time points (treatment start, and one, three, and twelve months after). The first US examination was performed within 3 days after treatment start. Study outcomes were clinical remission (defined as CDAI<150 after 12 months of treatment start) and treatment failure (defined as a change in medical therapy > 1 month after treatment start) during the follow-up period.

Paper II and **III** were designed as prospective cross-sectional studies, comparing the diagnostic accuracy of GIUS in predicting and quantifying endoscopic activity. All

patients were examined with US within two weeks before or after the ileocolonoscopy.

3.3 Ethical permissions

The Regional Ethics Committee for Medical and Health Research in Western Norway (REC West) approved all studies (REC West nos. 22209 (study I) and 2017/1750 (study II and III)). Studies II and III were reported to ClinicalTrials.gov ID: NCT03481751. Each study was performed in accordance with the Declaration of Helsinki. All patients gave written informed consent prior to participation.

3.4 Clinical assessment

Patient demographics, past medical history, and phenotype according to the Montreal classification (Appendix I) were obtained through patient anamnesis or, upon consent, access to the medical records. The Crohn's Disease Activity Index (CDAI) was used to evaluate clinical disease activity in **paper I**. However, the CDAI is complex and cumbersome to use, requiring a seven-day patient diary making it prone to recall bias. Therefore, Harvey Bradshaw Index (HBI) was used in **paper II** and **III**, enabling a simpler calculation of clinical disease activity. CDAI and HBI are presented in appendix II and III, respectively.

3.5 Biochemical analysis

Blood and stool samples were obtained within one week prior to, after, or on the same day as the US examination in all papers. Hemoglobin (g/dL), leucocyte count (10^9/L), platelet count (10^9/L), CRP (mg/L), erythrocyte volume fraction, and

albumin (g/L) were analyzed from blood samples, while fecal calprotectin (mg/kg) was measured from stool samples. All biochemical samples were analyzed at the laboratory facilities of Haukeland University Hospital (paper I-III) and Ålesund Hospital (paper III).

3.6 Reference standard

In **paper I**, CDAI was used as reference standard for assessing disease activity where a CDAI score exceeding 150 points was considered as active disease, while a CDAI <150 points was defined as clinical remission. Decision to treat was based on a clinical consensus. In **paper II and III**, the Simple Endoscopic Score for Crohn's Disease (SES-CD) evaluated by ileocolonoscopy was used as reference standard. Endoscopic remission was defined as a SES-CD score of 0 and 0-2 in **paper II** and **III**, respectively. The SES-CD is presented in appendix IV.

3.7 Ultrasound methods

3.7.1 Ultrasound scanners and probes

A GE Logiq E9 high-end scanner (GE Healthcare, Milwaukee, USA) was used in all studies. The ultrasound scanners were equipped with low-frequency curvilinear probes (C1-5/C1-6, 1-6 MHz) and high-frequency linear transducers (9L, 5.5-9 MHz, and ML6-15, 9-15 MHz). The CEUS examinations were performed using a high-frequency linear probe (9L, 5.5-9 MHz). Further details regarding US equipment are provided in the enclosed papers.

3.7.2 B-mode examination

In all papers, the settings of frequency, focus, and gain were optimized until the best images were obtained. Each patient was examined with a low-frequency curvilinear

probe for overview and a linear transducer for a detailed examination of the bowel wall. Ultrasound scanning was performed as previously described (125, 145). In short, the large bowel was examined by scanning systematically from the terminal ileum and further distally in longitudinal section. As the remaining part of the small bowel is difficult to track, a systematic scanning of the four abdominal quadrants aiming for target lesions was performed. The examination of the rectum was performed using the convex probe as it is deeply located. All bowel wall thickness measurements were performed in the anterior wall in longitudinal section. Wall thickness was measured from the interface echo between the serosa and the proper muscle to the interface echo between the mucosa and the lumen, and two and three representative measurements were averaged in **paper II-III** and **I**, respectively.

In **paper I**, pathological wall thickness was defined as >2 mm if the bowel lumen diameter was >0.5 cm and >3 mm if the lumen diameter was <0.5 cm or collapsed. Additionally, the thickness of individual wall layers was measured. In **paper II** and **III**, pathological wall thickness was defined as >3 mm.

The length of the affected segments was measured in **paper I** and **III.** In **paper II** and **III**, color Doppler measurements were recorded and quantified in segments with pathological wall thickness. Moreover, focal or entire disruption of bowel wall stratification and the presence of fatty wrapping were evaluated in **paper III**. Finally, the presence of stenosis and fistulas were recorded during the first part of **paper III**. Further definitions and score characteristics of the ultrasound variables are presented in appendix V.

3.7.3 Doppler examination

In **paper II** and **III**, color Doppler was performed on bowel segments exceeding 3 mm. Doppler settings were adjusted for optimal registration of low blood flow velocities. The velocity scale was reduced to 5 cm/s while gain was increased until

flash artifacts occurred and then lowered until they disappeared. The acquisitions were performed during patient breath-hold to reduce motion artifacts. Color pixels were interpreted as vessels if they persisted during the observation period. Bowel wall vascularity was evaluated semi-quantitatively by counting the number of Doppler signals per cm^2 using a modified version of (150), where 0-1, 2-5, and >5 signals were scored as 0, 1 and 2, respectively (appendix V). In **paper II**, a Doppler score of 0 was interpreted as remission, while activity was defined as a Doppler score of 1-2.

3.7.4 Software for interobserver assessment

In **paper III**, still images and cine loops of patients included in the development cohort were reviewed by another examiner to assess interobserver reproducibility of the chosen sonographic parameters. The software evaluation was performed on the development cohort before including the validation cohort. We used two offline software applications: Phillips DICOM Viewer (Phillips Medical Systems, Best, The Netherlands) and Onis® (DigitalCore, Co. Ltd, Tokyo, Japan). The application from Phillips was used for most purposes due to its simplicity and reliability, while the evaluation of Doppler signals was performed using the Onis viewer as it enables measurements of cm^2.

3.7.5 Contrast-enhanced ultrasound

In **paper I**, we performed contrast-enhanced ultrasound. In the study preset, the Logiq E9 uses amplitude modulation to register UCA backscattering. General contrast settings were selected, the gain adjusted to reduce tissue-derived signals, and the MI was set to 0.09-0.12 to prevent bubble destruction. Sonovue (Bracco, Milan, Italy) was used as UCA in all examinations.

A peripheral venous catheter of 20 gauge (1.1 mm) was inserted in the left cubital

vein, through which the UCA was administered. The contrast-injection was performed by a hospital nurse instructed beforehand. The anterior wall at the thickest section observed during the B-mode scan was examined with CEUS in longitudinal view.

The CEUS examination was performed using the Bolus tracking technique. In each patient, two contrast injections were performed consecutively, and 60-second acquisitions were made over the right iliac artery and the affected bowel loop. The CEUS data was saved as a Digital Imaging and Communications in Medicine (DICOM) file. Further details of the CEUS-examination are described in the corresponding paper.

3.7.6 Software for perfusion analysis

The CEUS data was evaluated, re-linearized, and quantified using a commercially available software application, VueBox® (Bracco Suisse SA, Geneva, Switzerland, version 4.2), as described in **paper I.** The program fits the time-intensity data to a standardized curve from which different perfusion parameters are derived.

However, most perfusion parameters are presented as arbitrary units, relative to the actual perfusion. To solve this issue, we performed a scaling procedure of the bowel parameters using the right iliac artery as an internal reference. The time-related parameters are not influenced by the concentration of the UCA (194), making the scaling procedure unnecessary.

Three parameters were excluded before final analysis; *Time of arrival* and *time to peak* are significantly influenced by the arterial input factor (AIF) and were thus avoided. The *wash in perfusion index* is calculated from other parameters and does not provide additional information.

3.8 Statistics

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) (IBM, Inc Armonk, NY), version 20 and 25 for **paper I** and **II-III**, respectively.

Demographical data are presented as median, minimum, and maximum values in all studies. The distribution of the data set was evaluated by inspecting histograms and boxplots as well as using the Shapiro-Wilk test. For continuous data, comparison between patient groups was performed using Student's t-test if normally distributed, and Mann-Whitney U test if not. For categorical data, the Chi^2-test or Fischer exact test were used. Spearman's rank was used to describe the correlation between different variables.

In **paper II**, the diagnostic accuracy of GIUS and clinical- and biochemical tests were expressed by sensitivity, specificity, positive predictive value, negative predictive value, and total accuracy. Furthermore, kappa statistics were used to evaluate inter-rater reliability as well as to investigate the agreement between ileocolonoscopy and clinical- biochemical- and ultrasonographic variables.

In **paper III**, multiple linear regression was performed to select which ultrasound parameters that should be included in an ultrasonographic scoring system. Spearman's rank and intra-class correlation (ICC) were used for assessing inter-rater correlation and agreement, and limits of agreement and assessment of potential biases between the investigators were evaluated using Bland-Altman analysis. Furthermore, a receiver operator curve analysis was performed to identify suitable cut-offs for separating remission and activity.

The level of significance was $P<0.05$ in all papers. Further details regarding statistics and data handling are presented in the included papers.

4. Summary of main results

4.1 Paper I

14 patients were included of which six had treatment failure during the follow-up period. At 12 months, 11 patients were in clinical remission, two patients had active disease, and one patient underwent surgery. There were no statistically significant differences between the treatment failure and effective treatment groups in demographics, clinical- or biochemical data at any time point.

We found significant differences in some amplitude-related perfusion parameters one month after treatment start: peak enhancement ($p=0.013$), wash-in area under the curve ($p=0.013$), wash-in rate ($p=0.020$), and wash-out rate ($p=0.008$). These differences occurred before changes in any treatment was done. There were no significant differences for the remaining amplitude-related parameters at one month (wash-out area under the curve ($p=0.142$) and wash-in/ wash-out area under the curve ($p=0.059$)), or at the other time points during follow-up (at 0, 3 and 12 months). The time-related parameters were statistically insignificant at each time-point.

There were no significant differences in BWT or length of the affected segments at any time point. However, we found significant differences in bowel wall layers, where the proper muscle- and submucosal layers were significantly thicker in non-responders at one and three months after treatment initiation, respectively.

4.2 Paper II

102 patients had active disease and 43 patients were in endoscopic remission. There were significant differences between the groups in disease behavior and previous surgery, as well as for HBI, CRP, and calprotectin.

A bowel wall thickness (BWT) exceeding 3 mm provided a sensitivity and specificity of 92.2% and 86% to distinguish between patients with inflammatory activity and patients in remission. Corresponding values for color Doppler were 66.7% and 97.7%, respectively. Moreover, BWT ($r=0.69$, $p<0.001$) and color Doppler ($r=0.64$, $p<0.001$) correlated well with SES-CD. Furthermore, an interobserver analysis of a subset of the included patients (n=23) revealed excellent agreement between the investigators for both BWT ($k=0.90$) and color Doppler ($k=0.91$) measurements. The diagnostic accuracy of ultrasonography significantly exceeded the corresponding values for clinical- and biochemical tests.

False negative results (n=8) were due to aphthous lesions in the terminal ileum in five cases (SES-CD=3), aphthous lesions and edema in the terminal ileum (SES-CD=6) were present in one case, while two cases revealed erythema and faded vascular pattern in the colon (SES-CD=1 and 6). Six false positive results were present in both colon (n=2) and the terminal ileum (n=4). By adding fecal calprotectin in isolated colonic thickening, all patients were correctly classified.

4.3 Paper III

A total study population of 164 were prospectively included, of which 40 and 124 were included in the development and validation phases, respectively.

Due to significant multicollinearity between BWT and length as well as no cases of fistulas, we excluded the latter two parameters. The combination of the remaining parameters provided the highest multiple correlation coefficient ($r=0.78$), but the predictive value of the ultrasound score was not reduced after excluding stenosis.

By re-examining the development cohort, we found good to excellent agreement between the investigators for calculating the score (ICC=0.93), as well as for BWT (ICC=0.91) and color Doppler (ICC=0.94). However, a poorer agreement was revealed for evaluating stratification (ICC=0.60) and fatty wrapping (ICC=0.45).

There were no fixed or proportional biases between the investigators in assessing the activity index and its components.

BWT and color Doppler were the only parameters with unique significant contributions to the model, and they achieved high interobserver reliability. Thus, we therefore simplified the ultrasound score by excluding stratification and fatty wrapping. The remaining activity index correlated well with SES-CD ($r=0.83$, $p<0.001$) and had high reproducibility (ICC=0.95)

The simplified ultrasound score correlated well with ileocolonoscopy ($r=0.78$ $p<0.001$) in the validation cohort, while poorer correlations were revealed for clinical- and biochemical tests. 23 patients were independently examined by two investigators, revealing excellent agreement (ICC=0.90) with no fixed or proportional bias.

5. Discussion

5.1 Methodological considerations

In **paper I**, we used the CDAI as reference standard as it is commonly employed to define clinical endpoints in trials. Still, assessment of clinical disease activity remains challenging as patients' symptoms do not reliably measure underlying inflammation (49), and current treatment goals have shifted from alleviation of symptoms to objective endpoints. Although developed for measuring disease activity in CD, similar CDAI scores may be present in patients with Irritable Bowel Syndrome (IBS) (195), and due to a considerable coexistence of IBS in CD patients (196), the ability of clinical scores to distinguish between active CD and other conditions is limited. However, the decision to treat and changing treatment regime was performed as part of standard care by the treating physician unaware of the ultrasound results.

Ultrasound is operator-dependent, and the investigator's level of experience may significantly impact the quality of the results. All CEUS-acquisitions were obtained by an experienced sonographer in **paper I**, while the primary investigator conducted the perfusion analyses and was at that time a medical student with little experience in clinical ultrasound. Despite some training in bowel ultrasonography and instructions on how to use the quantification software, the lack of experience might influence the validity of the results. Other limitations in **paper I** was the small number of included patients and that no interobserver analyses were performed. Quantification of CEUS-derived perfusion holds several limitations which are further discussed in the following paragraphs.

In **paper II** and **III**, the primary investigator gained more experience during the inclusion period, which could affect the validity of the results. Still, all ultrasound examinations were under close supervision by experienced sonographers during the first months of inclusion. Thereafter, a second observer re-examined a randomly

selected subgroup of patients for interobserver assessment.

5.2 Discussion of the main results

5.2.1 Clinical and biochemical evaluation of disease activity

Clinical and biochemical markers seem appealing for monitoring activity as they are simple, non-invasive, and have low operator dependency. Although well-established and valuable in the clinic, clinical symptoms and CRP have limited reliability for assessing inflammatory activity in CD (49, 54). This was further clearly demonstrated in **paper II** and **III**, as these parameters yielded poor accuracy for separating patients in remission from activity and had poor correlation with endoscopic activity.

Fecal calprotectin is useful for initial work-up and follow-up examinations (17, 42). Still, it is probably better suited for evaluating distal inflammation, as seen in ulcerative colitis (54) and colonic CD (60). In **paper II** and **III**, we found poor sensitivity and only moderate correlation to endoscopy. Similar correlations between endoscopy and clinical- and biochemical tests are previously reported (50). By excluding patients with terminal ileitis, the diagnostic accuracy significantly improved. Thus, our findings confirm that calprotectin is better suited for evaluating activity in patients with colonic involvement. The ideal use of calprotectin in CD management is monitoring activity over time. Repeated samples from the same patients help identify changes in disease activity but patient reluctance for providing repeated samples (197, 198) limits its utility. This problem was clearly demonstrated in our studies, as patient compliance on delivering fecal samples was poor with 33-40% missing data. There were no significant differences between the groups for delivering fecal samples. Furthermore, although conflicting evidence exits (199), previous studies report intra-individual variability of calprotectin (200, 201) which may further complicate interpretation. Ultimately, although being important non-invasive tools in CD management, neither calprotectin nor CRP had sufficient

accuracy to predict endoscopic activity or remission and cannot replace ileocolonoscopic evaluation (50, 202).

To overcome some of the practical challenges of fecal sampling, development and validation of a blood-based multi-marker test was recently performed (203). The authors found that the test could discriminate between active disease and remission, suggesting that the biomarker could replace some ileocolonoscopies. Still, most biomarkers constituting the test are not routinely measured. Moreover, as it cannot depict neither site nor extent, it must be complemented by additional methods.

5.2.2 Ultrasonographic prediction of endoscopic activity

In **paper II and III**, we found that increased bowel wall thickness (> 3 mm) has high diagnostic accuracy in distinguishing patients with active disease from patients in endoscopic remission in a heterogeneous hospital cohort.

Although endoscopic remission is commonly considered as SES-CD of 0-2 (71), patients' long-term prognosis seems to improve when there is no evidence of macroscopic inflammation defined as SES-CD or CDEIS scores of 0 (204, 205). This may be of particular importance in patients eligible for treatment discontinuation as the relapse rate seems to be less in patients obtaining endoscopic- rather than clinical remission after therapy withdrawal (206). Thus, in **paper II**, we used such strict endoscopic criteria (SES-CD=0) which have not been previously compared to ultrasonography.

According to our data, GIUS seems to provide high sensitivity and positive predictive value for detecting inflammatory lesions and may be sufficient to evaluate disease activity in scenarios where continuation or escalation of treatment is appropriate. The diagnostic accuracy could be further improved by adding color Doppler on pathological bowel segments and fecal calprotectin in sonographic colitis. For patients eligible for treatment discontinuation, however, ultrasonography does not seem to provide sufficient accuracy as it is not sensitive enough to detect mild

inflammatory lesions and should thus be examined with ileocolonoscopy. Consequently, implementation of bowel ultrasound has the potential to reduce the number of ileocolonoscopic examinations, improving allocation of endoscopic resources, and lessen patients' need to undergo invasive procedures. The upcoming national screening program for colorectal cancer will demand more endoscopy resources and may further push the development for endoscopic surrogate markers in IBD care.

Our principal finding seems to be in concordance with other studies (131, 151, 163, 207, 208), although there are some differences in design, ultrasound thresholds, and reference standard. Increased BWT due to inflammatory activity is considered the most important ultrasound parameter to detect active CD (126). Moreover, a recent study found that BWT was the best ultrasound parameter for measuring disease activity with good discriminative ability as well as a high correlation with SES-CD (r=0.60) (208), similar to our findings. By adding color Doppler, the positive predictive value increased, but we did not reveal adequate negative predictive value. These results are consistent with previous reports (151, 159), and could be due to insensitivity of equipment, obesity, or measuring at increased depths (125). A recent study suggests that further evaluations with CEUS may be useful to determine disease status when Doppler signals are lacking (151). Still, these measurements are usually performed on bowel segments with increased BWT, thus the pre-test probability for activity increases. Hence, measurements of BWT seems most suited to decide whether patients are in remission or not, while Doppler and CEUS are useful to quantify disease activity.

Although increasing BWT correlates with disease severity (209), bowel wall thickening could also appear in fibrotic segments (210). Additional sonographic findings could be useful to distinguish between these entities; inflammatory segments could be depicted as loss of stratification, prominent submucosal layer, and increased

Doppler signals, while preserved stratification and thickened proper muscle layer suggests fibrosis (138, 139, 141, 151). In **paper I,** we found significantly thicker proper muscle and submucosal layers in the treatment failure group one and three months after treatment start, respectively. Such differences were not revealed for BWT which could be explained by a limited number of included study participants. Although reaching significance at one month only, a closer inspection of the proper muscle boxplots suggests that there were differences between the groups at treatment start and at three months as well. As a thickened proper muscle layer may be indicative of fibrosis (139), this could partly explain the lack of medical effect in our study. Furthermore, a thickened submucosal layer is associated with active CD (138, 139, 211), which corresponds to our results. Thus, measurements of individual wall layers are simple and may provide additional guidance for disease activity evaluation. Although promising, their clinical significance is poorly investigated and should be examined in larger studies. Emerging methods such as CEUS (139, 164, 166) and elastography (212) may potentially aid further differentiation, although methodological challenges limit current use.

Although the usefulness of ultrasonography is thoroughly demonstrated (132, 151, 177), incorporation into clinical practice in Norway is limited. Ultrasound is commonly perceived as subjective and highly operator-dependent thus limiting its clinical utility. Although conflicting evidence exists (137), BWT measurements are found to have good reproducibility (136, 213), in line with our results. Recent recommendations regarding measurement standardization and minimum training may aid to standardize acquisition and interpretation of the US findings (125, 214).

5.2.3 Ultrasonographic activity index to measure endoscopic activity

A recent expert review advocates the use of cross-sectional imaging for monitoring CD patients (215). Although an MRI-based approach accurately depicts disease activity (86, 216), most protocols require bowel preparation and distention, as well as

administration of intravenous contrast agents. Gadolinium-based contrast agents may accumulate in brain tissue and should be limited, although no harmful effects are currently proven (217). Diffusion-weighted MRI or the simplified MaRIA score may overcome some of the obstacles of conventional MRI, as they are less time-consuming and do not require administration of contrast agents (90, 218). Still, frequent use of MRI is limited due to the reduced availability and high costs.

Ultrasonography seems well suited for systematic activity monitoring of CD patients, as it is rapid, non-invasive, well-tolerated by patients, and feasible in out-patient clinics (132, 177, 198). An accurate ultrasound score may ease interpretation of sonographic activity, thus facilitating incorporation in clinic. Moreover, as it is useful to monitor the same patient over time, an ultrasound score can determine whether the inflammatory activity increases or decreases. Several ultrasound activity indices have previously been developed (146-148, 219-221), but most with inadequate methodology (178).

In **paper III**, we developed and validated a simple and reproducible ultrasound scoring system for Crohn's disease, overcoming the limitations of previous scoring systems. The activity index, the Simple Ultrasound Score for Crohn's Disease (SUS-CD), correlates well with the SES-CD and may thus be a surrogate of endoscopic activity. The usefulness in daily life is further demonstrated, as real-world data with patients at different disease stages were included.

Although BWT seems to be sufficient to distinguish between patients with active disease from patients in remission, it should be accompanied by additional sonographic parameters for quantifying activity. The SUS-CD was developed similarly as the SES-CD (66), using multiple linear regression to select the ultrasound parameters that should be included. Initially, seven sonographic parameters were carefully selected and weighted according to current knowledge (126). Length of the affected segment and stenosis were excluded due to multicollinearity and minimal unique contribution to the model, respectively. No case of fistula was present, and the

parameter was thus excluded. Although penetrating behavior indicates severe disease, the presence of fistulas or abscesses do not seem useful for score development as activity monitoring should be applicable in heterogeneous patient populations.

The interobserver analysis of the development cohort revealed excellent agreement for BWT (ICC=0.91) and color Doppler (ICC=0.94), while it was poorer for stratification (ICC=0.60) and fatty wrapping (ICC=0.45). A recent international inter-rater agreement study revealed similar findings where BWT (ICC=0.91) and color Doppler (κ=0.60) revealed good to excellent agreement, while stratification (κ=0.39) and fatty wrapping (κ=0.50) were less reproducible (213). These findings are in concordance with a previous Italian study (136). Still, poorer results are previously presented (137), highlighting the need for clear definitions and standardization of measurements. The high reproducibility of BWT and color Doppler could be due to their quantitative interpretation, while stratification and fatty wrapping are more subjective and thus more prone to different interpretations.

The SUS-CD (Appendix V) was finally constituted by BWT and color Doppler as they provided significant contributions in predicting endoscopic activity and was easy to reproduce. These parameters are the most commonly selected in score development (178) and seem to be the best reflectors of disease activity. By excluding complications, length, stratification, and fatty wrapping, the ultrasound score lacks the ability of evaluating further important aspects of CD. However, the trade-off yields a reliable, reproducible, and easy-to-use tool during follow up. The excluded parameters may instead serve as additional modifiers when present. Further discussion regarding parameter selection, significance, and interpretation is provided in the associated paper.

The ultrasound score seems well suited for monitoring CD activity, still, it is not developed for assessing proximal bowel segments, it may not be useful in patients with obesity or bowel gas, and does not seem to achieve sufficient sensitivity in detecting mild inflammatory lesions. Furthermore, increased BWT could occur in fibrotic segments as well, which could lead to misinterpretation. Thus, as the

ultrasound score may not be applicable in all patients, a careful selection of patients could be necessary. Also, it has not been tested for its ability to detect changes in disease activity. Although ultrasonography may not replace ileocolonoscopic examinations, it could serve as an adjunct. As it can be frequently performed, GIUS might facilitate close monitoring of disease activity and treatment response. Implementation of GIUS in clinic may potentially enable better allocation of endoscopic- and imaging resources.

5.2.4 Predictive value of bowel perfusion in CD

In **paper I**, we found that CEUS-derived bowel perfusion enables prediction of treatment outcome as there were significant differences between responders and non-responders one month after treatment start. Increased bowel perfusion due to angiogenesis and dysfunctional regulation of blood supply are features of active CD (38, 39), and perfusion estimates may be potential surrogate markers. Our principal finding suggests that the efficacy of a treatment regime is poor in patients with sustained increased perfusion and that an early change in therapy could be beneficial. The current practice in our hospital is to assess the therapeutic outcome after three months of treatment start. Hence, implementation of CEUS may potentially accelerate treatment decisions, monitor treatment effect, decrease doctor's delay, and enabling better tailoring of patient care. To our knowledge, this was the first study to report early perfusion differences with repeated CEUS-examinations during 12 months of follow-up.

All amplitude-based perfusion parameters except wash-out area under the curve and wash-in/ wash-out area under the curve were statistically significant one month after treatment start. However, with a closer examination of the boxplots, there seems to be a group effect and the non-significant results could be explained by type 2 errors. Moreover, the acquisition lasted for 60 seconds which might be insufficient to

evaluate the wash-out of contrast agents, and longer time recordings may be necessary. This was later demonstrated by Quaia et al. (167, 169) who found significant differences in all amplitude related parameters between responders and non-responders, when extending the contrast acquisition period to 120 seconds in larger patient cohorts (n=50 and n=115). In line with our results, they could neither find significant differences between time-related parameters.

Our principal finding is in concordance with other studies (167-169, 222), even though there are differences in methodology. In our study, re-linearized bowel perfusion was normalized using the right iliac artery as an internal scaling factor. In contrast, other research groups evaluated the percentage change between perfusion at baseline and follow-up without using a scaling factor (167, 169, 223), measured video intensity in greyscale on log-compressed recordings (222), or assessed the pre-post difference in contrast enhancement on the same recording on log-compressed video data (168). Measurements of contrast intensity in decibel may be another method useful in clinic (161) although linear intensity data is the only mathematically valid approach for perfusion calculation (115).

Beyond the differences in methods and numerical values, all aforementioned studies reached similar conclusions. There are, however, serious concerns regarding reproducibility which could be partly due to vendor-specific detection of microbubbles and difficulties in obtaining raw-data (116, 224, 225), as well as inter-individual differences in the arterial input function (AIF). Thus, both the quantification method as well as the US machine- and settings must be identical in follow-up examinations. The AIF describes the input of contrast agents to the tissue of interest and is substantially influenced by injection speed and inter-individual differences in size, vascular system, and physiology (111, 226, 227). The AIF could be estimated using a complex mathematical model, as proposed by Jirik et al., enabling calculation of absolute perfusion (mL/min) (111).

As different ultrasound systems measure contrast signals differently, the comparison between various US vendors becomes difficult (228). Application of a calibration

procedure using phantoms (224) or by measuring absolute perfusion (111) may overcome these challenges. Furthermore, the size and selection of the region of interest could significantly influence the result, suggesting that strict criteria are needed (229). To facilitate implementation of CEUS in treatment monitoring, an international consensus regarding standardization of acquisition, perfusion quantification, and software selection is warranted.

6. Conclusion

We demonstrated that gastrointestinal ultrasound could accurately quantify inflammatory activity in CD. We developed and validated a simple ultrasound activity index that correlates well with ileocolonoscopy and has low interobserver variability. We have also provided evidence for the ability of ultrasonography to accurately differentiate between patients with disease activity from patients in endoscopic remission. Bowel wall thickness exceeding 3 mm is a simple and reproducible cut-off value, providing sufficient discriminative ability. Thus, implementation of ultrasonography in outpatient clinics could significantly impact clinical decision making. Furthermore, by adding ultrasound contrast agents, we demonstrated the ability of ultrasonography to provide prognostic information regarding treatment effect, as there were perfusion differences between medical responders and non-responders. Still, challenges remain before CEUS could be implemented as part of routine clinical practice.

7. Future perspectives

Ileocolonoscopy will still be necessary in the management of CD patients but has limitations that restrict its use. Implementation of ultrasonography during follow-up could reduce the need for ileocolonoscopic examinations, enabling better allocation of endoscopic resources. Future studies or expert recommendations should further designate dedicated scenarios where ultrasonography may be appropriate, and further clarify which should be reserved for other modalities.
We developed an ultrasound activity index (SUS-CD) that correlates well with endoscopy and may ease interpretation of ultrasonographic disease activity. Still, the scoring system should be validated by other groups and tested for responsiveness to changes in disease activity before incorporation in clinical practice. Future studies may further investigate the ability of the SUS-CD to guide treatment decisions. The clinical significance of transmural healing is uncertain, although it may represent a profound level of healing. This could be further clarified in large prospective studies including MRI, ultrasound, and ileocolonoscopy.

Point-of-care-ultrasonography (POCUS) allows for rapid diagnosis at the bedside and has emerged as a valuable tool in the emergency department. In bowel ultrasound, POCUS is an evolving concept and still in its infancy. As BWT is simple, reproducible, and highly accurate to discriminate between active and inactive bowel segments, it may provide clinicians with additional guidance in point-of-care settings at the out-patient clinic, and should be further investigated in future studies

Contrast-enhanced ultrasound has an emerging role in CD management. However, there is high variability in detection and quantification of contrast agents, making standardization and interpretation of perfusion measurements difficult. Thus, standardization, simplification, and improvement of software and quantification procedure are needed. Moreover, a comparison of different quantification procedures ultimately selecting the most appropriate in daily clinical practice should be performed. Finally, 3- and 4D ultrasound may overcome some challenges of CEUS,

including out-of-plane images, difficulties in motion correction, and assessment of absolute perfusion.

Many CD patients suffer due to impaired bowel function. The need for frequent invasive investigations adds to the patients' burden. Broader implementation of ultrasound as a disease monitoring tool may aid clinicians to perform better tailoring of patient care as well as alleviating patient's burdens.

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9. Appendix

Appendix I

Montreal classification

	Montreal Classification
Age at diagnosis	
<16 years	A1
17-40 years	A2
>40 years	A3
Disease location	
Ileal disease	L1
Colonic disease	L2
Ileocolonic disease	L3
Isolated upper disease*	L4
Disease behavior	
Non-stricturing and non-penetrating	B1
Stricturing	B2
Penetrating	B3
Perianal disease modifier†	p

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.
†"p" is added to B1–B3 when concomitant perianal disease is present.

Appendix II

Crohn's Disease Activity Index

All factors relate to the last seven days before the exam.

Category	Weighting factor	Subtotal
Number of liquid or soft stools in 7 days	x2	
Abdominal pain, sum of 7 daily ratings 0=none, 1=mild, 2=moderate, 3=severe	x5	
General well-being, sum of 7 daily ratings 0=well, 1=slightly below par, 2=poor, 3=very poor, 4=terrible	x7	
Number of listed complications (One point for each) • arthritis or arthralgia • iritis or uveitis • erythema nodsosum, pyderma gangernosum or apththous stomatitis • anal fissure, fistula or perirectal abscess • Other fistulas • Fever (>37,8 degrees Celcius)	x20	
Use of drug to reduce diarrhoea? 0=No, 1=Yes	x30	
Abdominal mass 0=none, 2=questionable, 5=definite	x10	
Hematocrit: Males: 47-Hct= Females: 42-Hct=	x6	
Body weight change $100x \frac{1-weight}{standard\ weight}$	x1	

Final Score (add subtotals)		

Interpretation:
Remission: CDAI <150
Mild: CDAI 150-219
Moderate: CDAI 220-450
Severe: CDAI >450

Appendix III

Harvey-Bradshaw Index of Crohn's Disease

Responses should be based on the 24-hour period preceding the visit

Category	Subtotals
General Wellbeing 0 = Very Well 1 = Slightly Below Par 2 = Poor 3 = Very Poor 4 = Terrible	
Abdominal Pain 0 = None 1 = Mild 2 = Moderate 3 = Severe	
Number of liquid or very soft stools daily	
Abdominal Mass 0 = None 1 = Dubious 2 = Definite 3 = Definite and Tender	
Extra-intestinal manifestations of CD ***(score 1 per item)*** ☐ Arthralgia/Arthritis ☐ Uveitis/Iritis ☐ Erythema nodosum ☐ Apthous ulcers ☐ Pyoderma gangrenosum ☐ Anal fissure ☐ Draining fistula (eg, perianal, enterocutaneous, rectovaginal) ☐ Perianal Abscess	
Final Score (add subtotals)	

Interpretation:

Remission: HBI <5
Mild: HBI 5-7
Moderate: HBI 8-16
Severe: HBI >16

Appendix IV

Simple endoscopic activity score for Crohn's Disease (SES-CD)

Variables	Ileum	Right colon	Transv. colon	Left colon	Rectum	Total
Ulcer size (0-3)						
Ulcerated surface (0-3)						
Affected surface (0-3)						
Stenosis (0-3)						
					Score	

Definitions of variables in SES-CD

Variables	0	1	2	3
Ulcer size	None	Aphthous ulcers (0-0,5cm)	Large (0,5-2cm)	Very large ulcers (>2cm)
Ulcerated surface	None	<10%	10-30%	>30%
Affected surface	Unaffected	<50%	50-75%	>75%
Stenosis	No	Single, passable	Multiple, passable	Not passable

Interpretation:

Remission: SES-CD 0-2
Mild: SES-CD 3-6
Moderate: SES-CD 7-15
Severe: SES-CD ≥16

Appendix V

Definitions of variables eligible for the ultrasound index:

Variables	0	1	2	3
Bowel wall thickness	<3.0 mm	3.0-4.9 mm or 4.0-4.9 mm (rectum)	5.0-7.9 mm	≥8.0 mm
Stenosis	No stenosis	Suspected (Thickened wall with narrow lumen)	Suspected several per segment	Suspected with prestenotic dilatation (>2.5 cm)
Length of affected segment	No affection	<5 cm	5-10 cm	>10 cm
Color Doppler score	No or single vessel	2-5 vessels per cm^2	>5 vessels per cm^2	
Stratification	Normal	Focal loss	Diffuse loss	
Fatty wrapping	Absent	Present		
Fistula	Absent	Present		

Simple ultrasound score of Crohn's Disease (SUS-CD)

Variables	Ileum	Right colon	Transverse colon	Left colon	Rectum	Total
Bowel wall thickness (0-3)						
Color Doppler score (0-2)						
					Score	

Errata for
Ultrasonographic evaluation of disease activity in Crohn's Disease

Fredrik Bjorvatn Sævik

Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

28/06-20 _____
(date and sign. of candidate)

30/06-20 _____
(date and sign. of faculty)

Errata

Page 12	Publication status of **paper II** and **III** has changed from *in revision* (paper II) and *under review* (paper III) to *accepted* in both papers.
Page 32	Misspelling: “possible” corrected to “possibly”
Page 34	Misspelling: “recommend” corrected to “recommended”
Page 47	Missing comma: “In **paper II** and **III**” corrected to “In **paper II** and **III**,”
Page 48	Grammatical correction: “were” corrected to “was”
Page 51	Misspelling: “well tolerated” corrected to “well-tolerated”
	Grammatical correction: “affected segment” corrected to “the affected segment”
Page 52	Grammatical correction: “interpretation” corrected to “interpretations”
	Grammatical correction: ”seems” corrected to “seem”
	Grammatical correction: “are” corrected to “is”
Page 54	Grammatical correction: “quantification” corrected to “the quantification”
Page 57	Grammatical correction: “restricts” corrected to “restrict”
	Grammatical correction: “a high” corrected to “high”
	Grammatical correction: “are” to “is”
Throughout the thesis	Misspelling: “Contrast enhanced ultrasound” changed to “Contrast-enhanced ultrasound”

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