

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Prospective qualitative and quantitative non-invasive evaluation of intestinal acute GVHD by contrast-enhanced ultrasound sonography.**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/141900> since

*Published version:*

DOI:10.1038/bmt.2013.65

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***

*Questa è la versione dell'autore dell'opera:*

*[Bone Marrow Transplant. 2013 Nov;48(11):1421-8. doi: 10.1038/bmt.2013.65.*

*Epub 2013 May 13.]*

***The definitive version is available at:***

*La versione definitiva è disponibile alla URL:*

*[<http://www.nature.com/bmt/journal/v48/n11/full/bmt201365a.html>]*

# Prospective qualitative and quantitative non-invasive evaluation of intestinal acute GVHD by contrast-enhanced ultrasound sonography

E Benedetti, B Bruno, G B McDonald, A Paolicchi, F Caracciolo, F Papineschi, M Pelosini, D Campani, S Galimberti and M Petrini

## Abstract

Intestinal acute GVHD (I-aGVHD) is a life-threatening complication after allografting. Non-invasive bed-side procedures to evaluate extension and treatment response are still lacking. We hypothesized that, during I-aGVHD, contrast-enhanced ultrasound sonography (CEUS) could detect microcirculation changes (MVC) of the bowel wall (BW) and help to monitor treatment response. We prospectively employed CEUS in 83 consecutive patients. Of these, 14 patients with biopsy-proven intestinal GVHD (I-GVHD) were defined as the study group, whereas 16 patients with biopsy-proven stomach GVHD (U-GVHD) without intestinal symptoms, 6 normal volunteers and 4 patients with neutropenic enterocolitis were defined as the control group. All patients were evaluated with both standard ultrasonography (US) and CEUS at the onset of intestinal symptoms, during clinical follow-up and at flare of symptoms. Standard US revealed BW thickening of multiple intestinal segments, useful to determine the extension of GVHD. CEUS showed MVC, which correlated with GVHD activity, treatment response, and predicted flare of intestinal symptoms. US and CEUS findings were superimposable at diagnosis and in remission. CEUS was, however, more sensitive and specific to identify subclinical activity in patients with clinical relevant improvement. These findings were not observed in the control groups. CEUS is a non-invasive, easily reproducible bed-side tool useful to monitor I-aGVHD.

## Introduction

Intestinal acute GVHD (midgut syndrome, I-aGVHD) is a major cause of non-relapse mortality following an allograft.<sup>1,2</sup> Diagnosis remains problematic for some patients with this pathology in the midgut. Diarrhea volume is generally used to determine the severity of the intestinal involvement, but its clinical reliability is highly limited.<sup>3,4</sup> Overall, non-invasive specific and sensitive techniques to diagnose intestinal GVHD (I-GVHD), to evaluate treatment response and to guide the duration of immunosuppression are still lacking. Standard transabdominal ultrasonography (US) has already been used for its diagnosis and clinical follow-up,<sup>5,6,7</sup> and, more widely, in a variety of other intestinal diseases,<sup>8,9,10</sup> including inflammatory bowel diseases.<sup>11,12,13,14</sup> Recent studies have highlighted neovascularization in the early stages of GVHD.<sup>15</sup>

We previously reported our experience on the use of contrast-enhanced ultrasound (CEUS) during I-aGVHD that showed contrast enhancement uptake in the bowel wall (BW).<sup>16</sup> Schreyer *et al.*<sup>17,18</sup> have also reported similar findings.

Standard US is routinely employed to evaluate both small and large intestines in a number of intestinal diseases. It is widely employed for both diagnosis and follow-up of inflammatory bowel diseases such as Crohn's disease, where intramural and extramural involvements can accurately be evaluated.<sup>19</sup> Although more rarely, it has also been used for assessing I-GVHD.<sup>20</sup>

In this study, we have evaluated the changes of the BW during GVHD using both standard US and CEUS, which is an easily reproducible ultrasound technique. Real-time microvascular imaging has recently been made possible by novel echo-contrast enhancing agents and low mechanical-index harmonic sonography. Moreover, CEUS has already been extensively used in active Crohn's disease in which the neovascularization of the small BWs has been described.<sup>21,22,23,24</sup> Importantly, recent studies have highlighted neovascularization in the early stages of GVHD.<sup>15</sup>

Here, we report the preliminary findings of our prospective study on the role of CEUS as a non-invasive qualitative and quantitative tool to evaluate extension and monitor I-GVHD. Overall, our aims were to evaluate if standard US could detect the extension of I-GVHD; if BW microcirculation changes (MVC) by CEUS correlated with clinical symptoms and treatment response; and, finally, if CEUS enhancement findings were similar to those seen in active Crohn's disease.<sup>25</sup>

## Patients and methods

### Patients

Between 2008 and 2012, we prospectively evaluated 83 consecutive patients admitted for an allogeneic transplantation, at the Division of Haematology, University of Pisa, Italy. All patients underwent a standard US of the abdominal organs, small and large intestines as part of the pre-transplant work-up. All patients who developed post-transplant diarrhea and/or vomiting and/or abdominal cramping underwent transabdominal US of the small and large intestine as described below. The study group consisted of 14/83 patients who developed biopsy-proven I-GVHD. The control group consisted of 16 patients who developed biopsy-proven U-GVHD without intestinal symptoms;<sup>26</sup> 4 patients who developed neutropenic enterocolitis (NEC) after allografting;<sup>27, 28</sup> 6 healthy volunteers without any documented diseases. Patient characteristics are summarized in Table 1. Median age was 50 years old (range 22–66 years).

**Table 1 - Diagnosis in the study and control group, description of conditioning regimen, GVHD prophylaxis, stem cell source, donor, and number and type of events occurred.**

Diagnosis	No. of patients	Conditioning	GVHD prophylaxis	Stem cell source	Donor	Type and no. of events
B-ALL	8	Cy/TBI	Cya + short-course MTX	PBSC	Sib (N=3), MURD (N=5)	I-GVHD (N=4), U-GVHD (N=3), NEC (N=1)
T-ALL	2	Cy/TBI	Cya + short-course MTX	PBSC	MURD (N=2)	I-GVHD (N=1), U-GVHD (N=1)
MM	6	Flu/TBI (N=2), Thiotepa/Cy/MEL (N=3)	Cya + MMF (N=2) Cya + short course MTX (N=4)	PBSC	Sib (N=3), MURD (N=3)	I-GVHD (N=1), U-GVHD (N=5)
MCL	3	Thiotepa/Cy/MEL	Cya + short-course MTX	PBSC	Sib (N=1), MURD (N=2)	I-GVHD (N=1), U-GVHD (N=2)
AML	8	BU/Cy	Cya + short-course MTX	PBSC (N=6), BM (N=2)	Haplo (N=2), sib (N=2), MURD (N=4)	I-GVHD (N=4), U-GVHD (N=2), NEC (N=2)
sAML	1	BU/Cy	Cya + short-course MTX	PBSC	Sib (N=1)	U-GVHD (N=1)
MF	3	BU/Cy	Cya + short-course MTX	PBSC	Sib (N=1), MURD (N=2)	I-GVHD (N=2), U-GVHD (N=1)
Follicular NHL	3	Thiotepa/Cy/MEL	Cya + short-course MTX	PBSC (N=2), BM (N=1)	MURD (N=3)	I-GVHD (N=1), U-GVHD (N=1), NEC (N=1)
Healthy volunteers	6	NA	NA	NA	NA	NA

Abbreviations: B-ALL = B-cell-ALL; Flu = fludarabine; I-GVHD = intestinal GVHD; MCL = mantle cell lymphoma; MEL = melphalan; MF = myelofibrosis; MM = multiple myeloma; MMF = mycophenolate mofetil; MURD = matched unrelated donor; NA = not applicable; NEC = neutropenic enterocolitis; PBSC = peripheral blood stem cells; PD = progressive disease; T-ALL = T-cell acute lymphoblastic leukemia; U-GVHD, upper gut acute GVHD; VGPR = very good PR. Median age 50 years (range 22–66 years).

After ruling out bacterial, fungal and viral infections by routine stool cultures and PCR analyses (for herpes viruses, adenovirus, EBV, rotavirus and norovirus), patients with persistent nausea, vomiting and diarrhea underwent endoscopy for histological assessment as previously described by Sale *et al.*<sup>3</sup> and modified by Epstein *et al.*<sup>29</sup> CMV infection was ruled out by immunostaining techniques. Clinical assessment and grading of GVHD was performed according to standard criteria.<sup>30, 31</sup> All patients and volunteers provided written informed consent and the study was approved by the Institutional Review Board according to the Declaration of Helsinki.

### Standard sonography methodology

Standard (B-mode) US was performed at bed-side with a portable sonographer (Esaote model My Lab 25, Esaote Italia, Florence, Italy) without any preparation, within 24 h from the onset of clinical GVHD symptoms. The entire gastrointestinal tract was submitted to a gray-scale ultrasound examination (B-mode US). The colon was examined from the cecum to the sigmoid colon. The entire small bowel was examined with particular attention to the last portion of the terminal ileum,

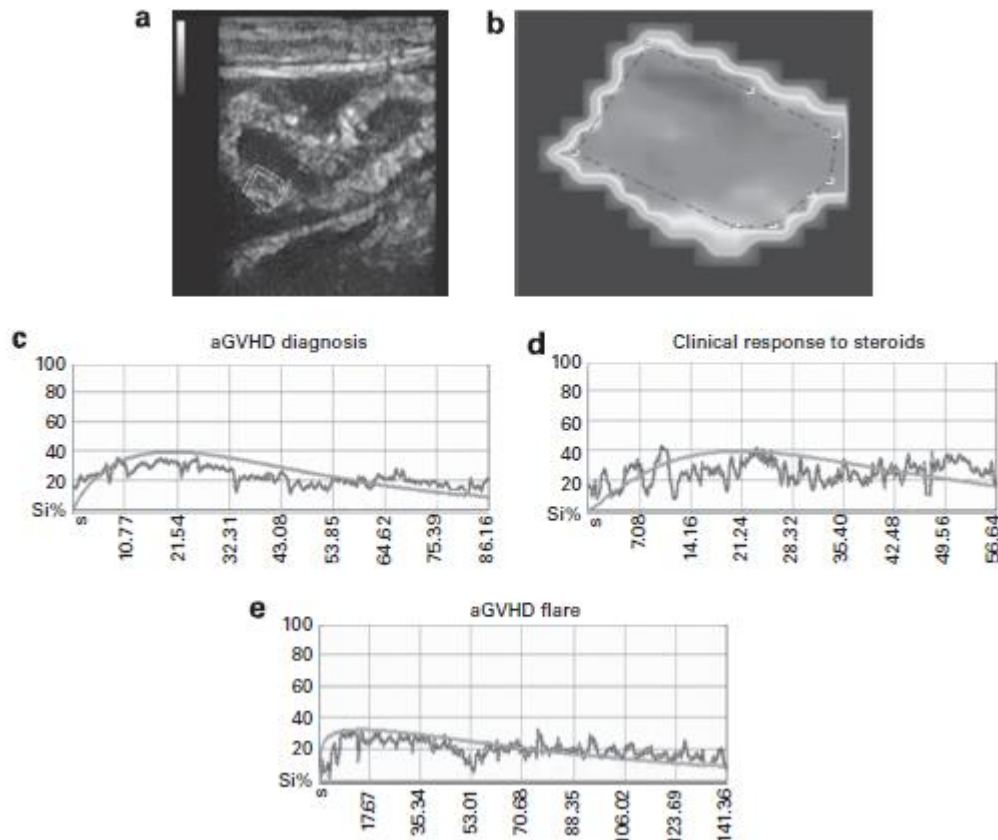
which is the site most commonly involved in GVHD.<sup>5, 6, 7</sup> The following parameters were assessed: (1) bowel wall thickness (BWT) defined as abnormal if  $\leq 3$  mm in the large bowel and  $\leq 2$  mm in the duodenum and small bowel;<sup>32</sup> (2) BW layers: the superficial mucosal interface, the deep mucosa, the submucosa, the muscularis propria and the serosa;<sup>33, 34</sup> (3) degree of dilation;<sup>32, 35</sup> (4) motility;<sup>32</sup> (5) bowel content defined as gas, food stuff or feces, mixtures of the two, or fluid-filled;<sup>36</sup> (6) presence of haustration or dehaustation;<sup>32, 37</sup> and (7) presence/absence of free abdominal fluid in all four quadrants and/or upper abdominal organ pathologies other than GVHD.

### **CEUS methodology**

After standard US, the ultrasound contrast agent was administered i.v. and CEUS was performed on diseased intestine<sup>38</sup> with the same sonographer equipped with contrast-specific real-time imaging technology defined as contrast tuned imaging. A second-generation echo-contrast agent, SonoVue (Bracco, Milan, Italy), was injected as i.v. bolus into an antecubital vein. Briefly, SonoVue is a non-nephrotoxic contrast agent that consists of 2.5- $\mu$ m-diameter microbubbles stabilized with phospholipids and filled with sulfur hexafluoride that flow through the pulmonary microcirculation and remain within the vascular space.<sup>39</sup> SonoVue is approved in Europe for clinical use and has a wide range of clinical applications.<sup>38, 40, 41</sup>

Contrast tuned imaging exploits the resonance property of the microbubbles and prevents them from bursting during insonation. This allows for real-time imaging of the microcirculation, without gray-scale echoes, and provides continuous perfusion data on viscera.<sup>25, 42</sup>

After injection the contrast agent reaches the intestinal wall in about 10–15 s and its peak concentration after approximately 30 s. In the intestine this ‘arterial phase’ is followed by the ‘venous phase’ in which the contrast agent, after distributing to the whole intestinal capillary bed, is exhaled through the lungs.<sup>38</sup> Continuous imaging was recorded from injection throughout the entire arterial and venous phases as previously described by Serra *et al.*<sup>25</sup> Distinct digital cine-clips for basic US and for CEUS scans were stored for computed analysis. Echo-signal intensity of the vascularity of the bowel segments selected with CEUS, defined by the operator as regions of interest (ROI) (Figure 1a), were quantitatively analyzed with a dedicated software (Q-ontrast; e-AMID—Advanced Medical Imaging Development, Italy distributed from Bracco). Q-ontrast generates chromatic maps (Figure 1b) of the ROI perfusion patterns, and automatically compensates for motion artifacts during data acquisition.<sup>43</sup> The Q-ontrast analysis of ROI generated curves representing echo-signal intensity vs time (time intensity curves). For all patients time intensity curve parameters, including the slope of the first ascending tract of the curve, the curve shape, time to peak enhancement, the area under the curve, regional blood flow and mean transit time were recorded (Figures 1c–e).<sup>43</sup>



**Figure 1.** (a) CEUS of an intestinal loop with aGVHD. The ROI is indicated by white arrow. (b) ROI quantitative analysis of BW vascularity with the Q-ontrast software with the corresponding color map (see text). (c) Representative TIC in Pt10 at I-aGVHD diagnosis. The curve has a ‘tailing’ shape with a mean transit time (MTT)=46.3 s and AUC=5.1 cm<sup>2</sup>. (d) Representative TIC in the same patient (Pt10) after clinical response to steroids: MTT and AUC decreased (MTT=38.7 s, AUC=0.25 cm<sup>2</sup>). (e) Representative TIC in Pt10 at I-aGVHD flare: MTT and AUC increased again (MTT=92.6, AUC=2.6 cm<sup>2</sup>). A full color version of this figure is available at the *Bone Marrow Transplantation* journal online. Full figure and legend (133K)

In all patients, US and CEUS were performed by a physician with 15 years of experience in US, who is a member and teacher of the Italian School of Basic and Emergency Ultrasound (SIUMB) at the University of Pisa.

### Statistical analysis

Sensitivity, specificity, positive and negative predictive values were determined to evaluate the diagnostic accuracy of standard US and CEUS. Their point estimates and 95% CIs were reported.

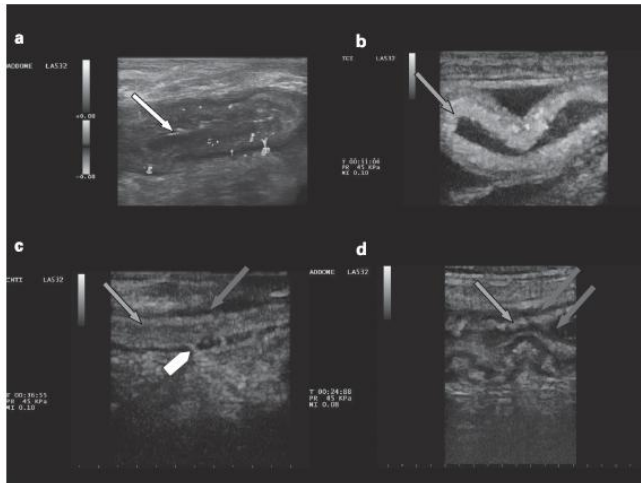
## Results

### Standard US findings of I-GVHD (study group)

Table 2 illustrates US and CEUS findings, and their correlation with GVHD and clinical outcomes. Standard US invariably revealed increased BWT mostly related to mucosal edema (Figure 2a).

Segments involved varied from patient to patient. Of 14 patients, 9 had more than one site involved

at the onset of symptoms. The BW layers could be identified in 11 patients, whereas in the others boundaries were poorly defined.



**Figure 2.** Imaging of aGVHD by color-doppler US and EPs (1, 2, 3) by CEUS. **(a)** Macrovascularization of the terminal ileum with color-doppler US (arterioles). Increased BWT (5.3 mm), mostly due to mucosal edema. An echo-rich layer is detectable between the mucosal layer and the bowel lumen of the distal ileum (white arrow). **(b)** EP 1: arterial phase enhancement of microcirculation with complete enhancement of the entire wall section from mucosal to the serosal layer (green arrow). **(c)** EP 2: the contrast medium reaches and enhances the BW microcirculation during the arterial phase (green arrows), with absence of enhancement only in the outer border of the muscularis propria (red arrow). The ‘Vasa recta’ are identified by contrast medium (white arrowhead). **(d)** EP 3: arterial phase enhancement of microcirculation showing absence of enhancement (red arrow) both in the outer and in the inner border of the BW and enhancement of microcirculation only in the intermediate submucosal layer (green arrow). A full color version of this figure is available at the *Bone Marrow Transplantation* journal online. Full figure and legend (123K)



**Table 2 - Standard US and CEUS findings at diagnosis, at follow-up and at flare.**

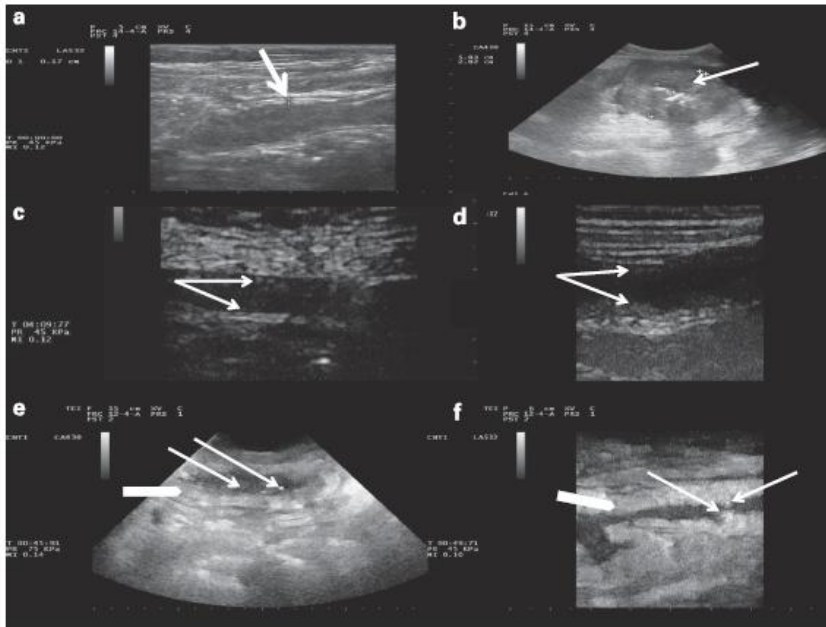
Pt	Findings at diagnosis				At 1st follow up				At 2nd follow-up			
	Standard US (involved sites, BWT in mm)	CEUS (site examined)	GVHD histology/clinical grade	Therapy and outcome	Salvage therapy	Standard US (involved sites, BWT in mm)	CEUS (site examined)	Therapy and outcome	Standard US	CEUS (site examined)	Therapy and outcome	
1	T. ileum (5.1); A.colon (5.8)	T. ileum: EP1 (AD)	Severe skin 3, Int 2 III	PDN, steroid refractory	Infliximab (2 doses)	Unchanged	T. ileum: EP1 despite CRI PAD	Continue infliximab (2 doses); CR	Normal after infliximab (4 doses)	Normal	Persistent CR	
2	T. ileum (7.9); A. colon(8.4); T. colon (4.2); D. colon (6.0)	A. colon: EP1 (AD)	Moderate Int 1 II	PDN CR	NA	Normal	Not done	CR				
3	A. colon (18); T. colon (7.6); D. colon (8.2); S. colon (15.1)	A. colon: EP3 (AD)	Moderate Int 3 III	PDN, steroid refractory	Infliximab (2 doses)	Unchanged	Unchanged PAD	Dead (MOF at D + 61)				
4	T. ileum (5.8); A. colon(6.0)	T. ileum: EP1 (AD)	Severe Int 4 IV	PDN, steroid refractory	Unchanged	Unchanged	Unchanged PAD	Dead (paralytic ileus D + 53) CR, D + 280 alive and well				
5	T. ileum (5.2)	T. ileum:EP2	Mild Int 1 II	PDN, CR	NA	Normal	Not done					
6	T. ileum (6.0); A. colon(6.2)	T. ileum: EP1 (AD)	Severe Int 4 IV	PDN, steroid refractory	Infliximab (2 doses)	Unchanged	Unchanged PAD	Continue infliximab (2 doses)	Unchanged	Unchanged PAD	Dead (sepsis D + 73)	
7	T. ileum (7.0); A. colon(5.5)	T. ileum: EP1 (AD)	Moderate skin 2, Int 3 III	PDN, steroid refractory	Infliximab (2 doses)	Improved	T. ileum: unchanged; JEP1 despite CRI, PAD	Continue infliximab (2 doses)	Unchanged	Unchanged PAD	Died (MOF D + 84)	
8	T. ileum (6.0)	T. ileum: EP2 (active disease)	Mild skin 2, Int 1 II	PDN, CR	Unchanged	Normal T. ileum (4mm)	Unchanged; T. ileum; JEP2 despite CRI, PAD	Budesonide	Normal	Normal	CR (alive D + 80)	
9	T. ileum (5.7); A. colon(5.5)	T. ileum: EP2 (AD)	Moderate Int 1 II	PDN, CR	Unchanged	Normal	Unchanged CR, died of HHV6-encephalitis D + 69	Unchanged CR, died of HHV6-encephalitis D + 69				
10	T. ileum (5.9); D. colon (5.5)	T. ileum: EP2 (AD)	Moderate skin 2, Int 1 II	PDN, CR	Unchanged	Normal	T. ileum: EP2 with CRI PAD	Budesonide	Normal	Normal	CR (D + 144)	
11	T. ileum (5.7)	T. ileum: EP2 (AD)	Moderate skin 2, Int 1 II	PDN, CR	Unchanged	Normal	T. ileum: JEP2, PAD	ECP Budesonide; CR	Normal	Normal		
12	A. colon (7.2); T. colon (6.5); D. colon (6.5)	D. colon: EP2 (AD)	Moderate Int 1 II	PDN, CR	Unchanged	A. and T. colon, normal; improved	D. colon: JEP2, PAD	Budesonide; CR	Normal	Normal	Persistent CR	
13	D. colon (6.1)	D. colon: EP2 (AD)	Moderate Int 1 II	PDN, CR	Unchanged	Unchanged	Unchanged PAD	ECP, Rituximab	Improved	Unchanged, persistent active disease	Dead (sepsis D + 46)	
14	D. colon (7.2)	D. colon: EP2 (AD)	Moderate Int 1 II	PDN, CR	Unchanged	Unchanged	Unchanged PAD	ECP, Rituximab	Normal	Normal	Dead (relapse D + 146)	
<b>Findings at flare</b>												
2nd follow-up												
1st follow-up												
Therapy and clinical outcome												
Standard US (involved sites, BWT in mm)												
CEUS (site examined)												
Therapy and clinical outcome												
Standard US (involved sites, BWT in mm)												
CEUS (site examined)												
Therapy and clinical outcome												
1	D. colon (7.5)	D. colon: EP2 (AD)	PDN CR	Normal	NF							
2	T. ileum (7.0)	T. ileum: EP2 CRI PAD	Rituximab (4 doses) CRI	Improved	T. ileum: EP2, CRI PAD							Persistent CR Overt flare; infliximab, dead (sepsis D + 223) CR; alive and well (D + 171)
10	T. ileum (5.2)	T. ileum: EP2 (AD)	PDN with CR	Normal	NF							

Abbreviations: A. colon = ascending colon; AD = active disease; AP = arterial phase; BW = bowel wall; BWT = contrast-enhanced ultrasonography; CRI = clinically relevant improvement; D. colon = descending colon; D + = day at follow-up; ECP = extracorporeal photophoresis; ENH = enhancement; EP1 = enhancement pattern 1 (Figure 1); EP2 = enhancement pattern 2 (Figure 2); EP3 = enhancement pattern 3 (Figure 3); Int = intestinal; MOF = multi-organ failure; MTT = mean transit time; NA = not applicable; NF = normal features; PAD = persistent active disease; PDN = prednisone; S. colon = sigmoid colon; T. ileum = terminal ileum; T. colon = transverse colon; Tx = transplant; ↓ = reduced. The table shows the correlation with aGVHD grade, stage, therapy, outcome and cGVHD. Underlined patients depict those in whom during follow-up there were different findings between CEUS and US.



### Standard US findings in the control group

Healthy volunteers ( $N=6$ ) and U-GVHD ( $N=16$ ) patients had normal BWT thickness (Figure 3a).<sup>8, 32</sup> On the contrary, patients ( $N=4$ ) with NEC showed BWT as for the study group (I-GVHD) (Figure 3b).<sup>27, 28</sup>



**Figure 3.** (a) Standard US of a normal volunteer showing normal BW thickness (BWT; 1.7 mm, white arrow). (b) Standard US in a patient with NEC showing BWT (28.2 mm, white arrow). (c) CEUS in a normal volunteer (control group) showing arterial phase enhancement of BW, and normal thickness. (d) CEUS in a patient with U-GVHD without I-GVHD (control group) showing arterial phase enhancement of BW, and normal thickness. White arrows show arterial enhancement. (e) CEUS with convex probe and (f) CEUS with high-frequency linear probe of the same patient with NEC showing BWT and arterial phase enhancement (white arrowhead). There is passage of microbubbles in the lumen (white arrows).

### CEUS findings in I-GVHD (study group)

CEUS was performed at diagnosis, during treatments and follow-up (Table 2).<sup>38</sup> CEUS was safely performed in all patients, including one with a baseline creatinine level of 4.5 mg/dL.<sup>40</sup> Overall, by Q-ontrast software analysis, a rapid enhancement peak, due to a rich microvascularization of the BW, described by the tailing-curve shape and a long tailing, expressed by a prolonged mean transit time was observed in all patients (Figure 1c).

### Findings at diagnosis

Overall, at aGVHD onset, during the arterial phase, CEUS showed three distinct enhancement patterns (EPs) of the bowel microcirculation: EP-1, a complete enhancement of the entire wall section from the mucosal to the serosal layer (Figure 2b); EP-2, enhancement of the entire wall with the exception of the outer border of the muscularis propria (Figure 2c); EP-3, enhancement of the intermediate submucosal layer without enhancement of the outer and inner borders of the BW (Figure 2d). Moreover, a persistent microcirculation enhancement longer than 2 min was seen during the venous washout in 11 of 14 patients.

## Follow-up findings

All 14 patients with GVHD were promptly treated with steroids at 2 mg/kg. Salvage treatment in steroid-refractory patients consisted of Infliximab,<sup>4, 44, 45</sup> Rituximab,<sup>46, 47</sup> extracorporeal photophoresis,<sup>48</sup> budesonide<sup>49</sup> alone or in various combinations (Table 2).

In patients who completely responded to treatment with normalization of standard US, CEUS showed similar findings as for healthy volunteers and U-GVHD patients (Figures 3c and d).<sup>38</sup> However, in patients with clinically relevant improvement but no complete response, CEUS showed persistent microvascular enhancement suggesting still active disease, while standard US showed normal findings. In steroid-refractory<sup>50, 51</sup> patients, CEUS became normal only in those who achieved complete remission after salvage therapy, whereas it remained unchanged in patients who eventually died of GVHD-treatment-related complications (Table 2). In patients with improvement but no complete remission, US showed a sensitivity of 55% (95% CI: 0.23–0.83) and specificity of 100% (95% CI: 0.19–1) with a positive predictive value of 100% (95% CI: 0.42–1) and negative predictive value of 38% (95% CI: 0.09–0.76), whereas CEUS showed a sensitivity of 100% (95% CI: 0.62–1) and specificity of 100% (95% CI: 0.62–1), with positive and negative predictive values of 100%.

## Findings at GVHD flare

Overall, I-aGVHD flared in three patients (Table 2, Figure 1e). The CEUS BW EPs correlated with clinical symptoms. Standard US and CEUS showed normal features in responsive patients. In one patient who only showed a clinical improvement, CEUS showed persistent microvascular changes suggestive of subclinical activity. Qualitative assessment of BW microcirculation enhancement for each patient during follow-up and flare was in accordance with quantitative assessment using time intensity curves. Patients responding to treatment had a decrease in mean transit time and area under the curve (Figures 1c–e).

## CEUS findings in the control group

CEUS showed a thin layer of enhancement of BW both in healthy volunteers (Figure 3c) and in U-GVHD control patients (Figure 3d). These findings differed from those in the I-GVHD study group (Figures 2b–d). In patients with NEC, there was a strong arterial phase enhancement, suggestive of BW microvascular changes due to infection-related inflammation (Figures 3e and f).<sup>27</sup> The US and CEUS findings in NEC patients were superimposable.

We found penetration of microbubbles in the bowel lumen, not only in the study group as previously reported,<sup>17, 18</sup> but also in one patient with NEC (Figures 3e and f).

## DISCUSSION

Acute GVHD and its complications are major causes of non-relapse mortality following an allograft. Given the non-specific symptoms and the significant side effects of intestinal GVHD treatment, a histological diagnosis via endoscopic biopsies is highly recommended and may be required in up to 40% of patients for differential diagnosis.<sup>52</sup> Reliable non-invasive procedures to evaluate its extension and treatment response are still lacking.<sup>3, 4</sup>

We hypothesized that CEUS could specifically detect MVC at the onset of GVHD and during its treatment. Our hypothesis partly derives from the observations that intestinal biopsies during acute GVHD show an increased microvessel circulation<sup>53</sup> and that graft-vs-host reactions are associated with increased neovascularization.<sup>15</sup> In our study, standard US mostly revealed abnormal BWT in multiple segments suggestive of extensive intestinal involvement, as shown in a previously published study that employed positron emission tomography (PET).<sup>54</sup> Increased BWT, due to mucosal edema, was consistently observed in both the small intestine and the colon as previously

described by Klein *et al.*<sup>5</sup> However, these non-specific findings are also found during several intestinal infections.<sup>27, 28</sup>

CEUS clearly showed a microcirculation enhancement of the BWs during the arterial phase, followed by a prolonged venous phase washout. The persistence of the contrast agent in the BWs was expressed by the prolonged mean transit time. Interestingly, the EPs observed were similar to those described in active Crohn's disease.<sup>25</sup> The tailing shape of the washout curve may be explained by a marked inflammatory interstitial edema that may correlate with the pericapillary hemorrhage described in biopsies during active GVHD.<sup>22</sup> The interstitial edema does not prevent the arterial in-flow but slows the circulation in the venules and is likely responsible for the prolonged venous phase washout.<sup>23</sup> Furthermore, GVHD pathophysiology is characterized by neovascularization, mainly driven by vasculogenesis, during its early inflammatory phase. At a later stage, the vasculature itself becomes a target of allo-reactive donor T cells leading to fibrosis and rarefaction of blood vessels.<sup>15</sup> Overall, the increased arterial microvascular enhancement may correlate with an early neovascularization of the BWs<sup>15</sup> and the prolonged venous phase washout may be seen where the inflammatory interstitial edema is more prominent.

GVHD response to treatments correlates with longer survival.<sup>17</sup> In our study, complete clinical remission, after first-line or salvage treatment, coincided with normalization of both standard US and CEUS. However, patients with clinically relevant improvement without complete resolution of symptoms are a challenging subgroup at high risk of flare. Close follow-up is mandatory. Standard US only allows monitoring of BWT. On the contrary, CEUS allows us to detect microvascular changes at a capillary level, underlying persistent inflammatory activity, also in patients with normal BWT. Though the patient series was small (14 patients in the study group and 16 in the control group), CEUS was significantly more sensitive and specific than standard US in identifying subclinical GVHD activity, predictive of clinical flare, in patients without complete resolution of symptoms. Similarly, Di Sabatino *et al.*<sup>24</sup> described a group of patients with clinically inactive Crohn's disease who showed enhanced CEUS signal intensity. This group of patients developed early relapse. The authors suggested that CEUS findings were suggestive of subclinical disease activity that preceded early relapse.<sup>24</sup> Moreover, Serra *et al.*<sup>25</sup> reported a significant correlation between CEUS EPs and Crohn's disease activity. These observations are similar to what we have seen in our I-GVHD patients with abnormal CEUS findings despite relevant improvement of symptoms. Moreover, US and CEUS allowed to rule out lower intestinal GVHD when patients only presented with U-GVHD. This could be particularly useful in U-GVHD patients who can be treated with lower doses of steroids, reducing their side effects, as compared with patients with midgut symptoms.

CEUS is well tolerated, non-invasive and less expensive than other imaging techniques.<sup>54</sup> Moreover, it is applicable to patients with renal insufficiency, where CT or MRI with contrast media are contraindicated.<sup>55</sup> The reported incidence of severe hypersensitivity or allergic events to CEUS contrast agents is extremely low (0.001%).<sup>40</sup>

In conclusion, though not diagnostic, CEUS showed MVC of the BW that correlated with clinical symptoms of biopsy-proven I-GVHD and its treatment response at diagnosis, at follow-up and at flare.

Importantly, even though US and CEUS findings were superimposable in patients at diagnosis and in complete clinical remission after treatment, CEUS was more sensitive and specific in identifying residual disease activity in high-risk patients with clinically relevant improvement but not complete resolution of symptoms.

Moreover, CEUS provided a quantitative measurement of altered vasculature in segments inaccessible to endoscopic evaluation. Larger prospective studies are warranted to establish the role of the combination of clinical signs and/or symptoms with CEUS findings in the clinical follow-up of biopsy-proven I-GVHD.

## Conflict of interest

The authors declare no conflict of interest.

## References

1. McDonald GB, Shulman HM, Sullivan KM, Spencer GD. Intestinal and hepatic complications of human bone marrow transplantation. Part I. *Gastroenterology* 1986; 90: 460–477. |
2. McDonald GB, Shulman HM, Sullivan KM, Spencer GD. Intestinal and hepatic complications of human bone marrow transplantation. Part II. *Gastroenterology* 1986; 90: 770–784.
3. Sale GE, Shulman HM, McDonald GB, Thomas ED. Gastrointestinal graft-versus-host disease in man. A clinicopathologic study of the rectal biopsy. *Am J Surg Pathol* 1979; 3: 291–299.
4. Fisk JD, Shulman HM, Greening RR, McDonald GB, Sale GE, Thomas ED. Gastrointestinal radiographic features of human graft-vs.-host disease. *Am J Roentgenol* 1981; 136: 329–336.
5. Klein SA, Martin H, Schreiber-Dietrich D, Hermann S, Caspary WF, Hoelzer D *et al.* A new approach to evaluating intestinal acute graft-versus-host disease by transabdominal sonography and colour Doppler imaging. *Br J Haematol* 2001; 115: 929–934.
6. Görg C, Wollenberg B, Beyer J, Stolte MS, Neubauer A. High-resolution ultrasonography in gastrointestinal graft-versus-host disease. *Ann Hematol* 2005; 84: 33–39.
7. Haber HP, Schlegel PG, Dette S, Ruck P, Klingebiel T, Niethammer D. Intestinal acute graft-versus-host disease: findings on sonography. *Am J Roentgenol* 2000; 174: 118–120.
8. Cammarota T, Sarno A, Robotti D, Bonenti G, Deboni P, Versace K *et al.* US evaluation of patients affected by IBD: how to do it, methods and findings. *Eur J Radiol* 2009; 69: 429–437.
9. Robotti D, Cammarota T, Deboni P, Sarno A, Astegiano M. Activity of Crohn disease: value of Color-Power-Doppler and contrast-enhanced ultrasonography. *Abdom Imaging* 2004; 29: 648–652.
10. Gritzmann N, Hollerweger A, Macheiner P, Rettenbacher T. Transabdominal sonography of the gastrointestinal tract. *Eur Radiol* 2002; 12: 1748–1761.
11. Maconi G, Imbesi V, Bianchi Porro G. Doppler ultrasound measurement of intestinal blood flow in inflammatory bowel disease. *Scand J Gastroenterol* 1996; 31: 590–593.
12. Giovagnorio F, Diacinti D, Vernia P. Doppler sonography of the superior mesenteric artery in Crohn's disease. *Am J Roentgenol* 1998; 170: 123–126.
13. Ludwig D, Wiener S, Brüning A, Schwarting K, Jantschek G, Stange EF. Mesenteric blood flow is related to disease activity and risk of relapse in Crohn's disease: a prospective follow-up study. *Am J Gastroenterol* 1999; 94: 2942–2950.
14. Mayer D, Reinshagen M, Mason RA, Muche R, von Tirpitz C, Eckelt D *et al.* Sonographic measurement of thickened bowel wall segments as a quantitative parameter for activity in inflammatory bowel disease. *Z Gastroenterol* 2000; 38: 295–300.
15. Penack O, Socié G, Van den Brink MRM. The importance of neovascularization and its inhibition for allogeneic hematopoietic stem cell transplantation. *Blood* 2011; 117: 4181–4189.
16. Benedetti E, Caracciolo F, Papineschi F, Bruno B, McDonald GB, Pelosini M *et al.* Contrast enhanced ultrasound sonography in intestinal acute graft-versus-host disease. *Bone Marrow Transplant* 2009; 43: S367–S367.
17. Schreyer AG, Landfried K, Zorger N, Hoffstetter P, Ammer J, Fellner C *et al.* Transmural penetration of intravenously applied microbubbles during contrast-enhanced ultrasound as a new diagnostic feature in patients with GVHD of the bowel. *Bone Marrow Transplant* 2011; 46: 1006–1011.

18. Schreyer AG, Landfried K, Jung EM, da Silva NPB, Poschenrieder F, Dornia C *et al.* Contrast-enhanced ultrasound for differential diagnosis of suspected GvHD in patients after allogeneic transplantation. *Clin Hemorheol Microcirc* 2011; 49: 129–136.
19. Maconi G, Parente F, Bollani S, Cesana B, Bianchi Porro G. Abdominal ultrasound in the assessment of extent and activity of Crohn's disease: clinical significance and implication of bowel wall thickening. *Am J Gastroenterol* 1996; 91: 1604–1609.
20. Klein SA, Martin H, Schreiber-Dietrich D, Hermann S, Caspary WF *et al.* A new approach to evaluating intestinal acute graft-versus-host disease by transabdominal sonography and colour Doppler imaging. *Br J Haematol* 2001; 115: 929–934.
21. Piscaglia F, Nolsøe C, Dietrich CF, Cosgrove DO, Gilja OH, Bachmann M *et al.* The EFSUMB Guidelines and Recommendations on the Clinical Practice of Contrast Enhanced Ultrasound (CEUS): update 2011 on non-hepatic applications. *Ultraschall Med* 2012; 1: 33–59.
22. Ertault-Daneshpouy M, Leboeuf C, Lemann M, Bouhidel F, Ades L, Gluckman E *et al.* Pericapillary hemorrhage as criterion of severe human digestive graft-versus-host disease. *Blood* 2004; 103: 4681–4684.
23. Pullinger BD, Florey HW. Some observations on the structure and functions of lymphatics: their behavior in local edema. *J Exp Pathol* 1935; 16: 49–61.
24. Di Sabatino A, Fulle I, Ciccocioppo R, Ricevuti L, Tinozzi FP, Tinozzi S *et al.* Doppler enhancement after intravenous levovist injection in Crohn's disease. *Inflamm Bowel Dis* 2002; 8: 251–257.
25. Serra C, Menozzi G, Labate AM, Giangregorio F, Gionchetti P, Beltrami M *et al.* Ultrasound assessment of vascularization of the thickened terminal ileum wall in Crohn's disease patients using a low-mechanical index real-time scanning technique with a second generation ultrasound contrast agent. *Eur J Radiol* 2007; 62: 114–121.
26. Gooley TA, Chien JW, Pergam SA, Hingorani S, Sorrow ML, Boeckh M *et al.* Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med* 2010; 363: 2091.
27. Gorschlüter M, Mey U, Strehl J, Ziske C, Schepke M, Schmidt-Wolf IGH *et al.* Neutropenic enterocolitis in adults: systematic analysis of evidence quality. *Eur J Haematol* 2005; 75: 1–13.
28. Benedetti E, Caracciolo F, Lippolis P, Bruno B, Caramella D, Cerri F *et al.* Neutropenic enterocolitis: prospective study on usefulness of ultrasound sonography for early diagnosis and to guide medical or surgical treatment. *Bone Marrow Transplant* 2012; 47: S77.
29. Epstein RJ, McDonald GB, Sale GE, Shulman HM, Thomas ED. The diagnostic accuracy of the rectal biopsy in acute graft-versus-host disease: a prospective study of thirteen patients. *Gastroenterology* 1980; 78: 764–771.
30. Thomas ED, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE. Success or failure of marrow engraftment and marrow-graft rejection (second of two parts). *N Engl J Med* 1975; 292: 895–902.
31. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ *et al.* National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* 2005; 11: 945–956.
32. Kuzmich S, Howlett DC, Andi A, Shah D, Kuzmich T. Transabdominal sonography in assessment of the bowel in adults. *Am J Roentgenol* 2009; 192: 197–212.
33. Bolondi L, Casanova P, Santi V, Caletti G, Barbara L, Labò G. The sonographic appearance of the normal gastric wall: an *in vitro* study. *Ultrasound Med Biol* 1986; 12: 991–998.
34. Lim JH, Jeong YM. Sonography of the stomach: an *in vitro* study to determine the anatomic cause of inner hyperechoic and hypoechoic layers of the gastric wall. *Am J Roentgenol* 1994; 162: 335–338.

35. Hollerweger A. Colonic diseases: the value of US examination. *Eur J Radiol* 2007; 64: 239–249.
36. Cartoni C, Dragoni F, Micozzi A, Pescarmona E, Mecarocci S, Chirletti P *et al.* Neutropenic enterocolitis in patients with acute leukemia: prognostic significance of bowel wall thickening detected by ultrasonography. *J Clin Oncol* 2001; 19: 756–761.
37. Hagi C, Badea R. Applicability of abdominal ultrasonography in inflammatory bowel disease. *J Gastrointest Liver Dis* 2007; 16: 205–209.
38. Piscaglia F, Nolsøe C, Dietrich CF, Cosgrove DO, Gilja OH, Bachmann M *et al.* The EFSUMB guidelines and recommendations on the clinical practice of contrast enhanced ultrasound (CEUS): update 2011 on non-hepatic applications. *Ultraschall Med* 2012; 1: 33–59.
39. Morel DR, Schwieger I, Hohn L, Terrettaz J, Llull JB, Cornioley YA *et al.* Human pharmacokinetics and safety evaluation of SonoVue, a new contrast agent for ultrasound imaging. *Invest Radiol* 2000; 35: 80–85.
40. Claudon M, Cosgrove D, Albrecht T, Bolondi L, Bosio M, Calliada F *et al.* Guidelines and good clinical practice recommendations for contrast enhanced ultrasound (CEUS)—update 2008. *Ultraschall Med* 2008; 29: 28–44.
41. Quaia E, Calliada F, Bertolotto M, Rossi S, Garioni L, Rosa L *et al.* Characterization of focal liver lesions with contrast-specific US modes and a sulfur hexafluoride-filled microbubble contrast agent: diagnostic performance and confidence. *Radiology* 2004; 232: 420–430.
42. Migaleddu V, Scanu AM, Quaia E, Rocca PC, Dore MP, Scanu D *et al.* Contrast-enhanced ultrasonographic evaluation of inflammatory activity in Crohn's disease. *Gastroenterology* 2009; 137: 43–52.
43. Quaia E, Migaleddu V, Baratella E, Pizzolato R, Rossi A, Grotto M *et al.* The diagnostic value of small bowel wall vascularity after sulfur hexafluoride-filled microbubble injection in patients with Crohn's disease. Correlation with the therapeutic effectiveness of specific anti-inflammatory treatment. *Eur J Radiol* 2009; 69: 438–444.
44. Deeg HJ. How I treat refractory acute GVHD. *Blood* 2007; 109: 4119–4126.
45. Patriarca F, Sperotto A, Damiani D, Morreale G, Bonifazi F, Olivieri A *et al.* Infliximab treatment for steroid-refractory acute graft-versus-host disease. *Haematologica* 2004; 89: 1352–1359.
46. Kharfan-Dabaja MA, Cutler CS. Rituximab for prevention and treatment of graft-versus-host disease. *Int J Hematol* 2011; 93: 578–585.
47. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, von Bergwelt-Baildon MS. The role of B cells in the pathogenesis of graft-versus-host disease. *Blood* 2009; 114: 4919–4927.
48. Perfetti P, Carlier P, Strada P, Gualandi F, Occhini D, Van Lint MT *et al.* Extracorporeal photopheresis for the treatment of steroid refractory acute GVHD. *Bone Marrow Transplant* 2008; 42: 609–617.
49. Ibrahim RB, Abidi MH, Cronin SM, Lum LG, Al-Kadhimi Z, Ratanatharathorn V *et al.* Non absorbable corticosteroids use in the treatment of gastrointestinal graft-versus-host disease. *Biol Blood Marrow Transplant* 2009; 15: 395–405.
50. Deeg HJ, Antin JH. The clinical spectrum of acute graft-versus-host disease. *Semin Hematol* 2006; 43: 24–31.
51. Bacigalupo A. Management of acute graft-versus-host disease. *Br J Haematol* 2007; 137: 87–98.
52. Thompson B, Salzman D, Steinhauer J, Lazenby AJ, Wilcox CM. Prospective endoscopic evaluation for gastrointestinal graft-versus-host disease: determination of the best diagnostic approach. *Bone Marrow Transplant* 2006; 38: 371–376.

53. Shidham VB, Chang CC, Shidham G, Ghazala F, Lindholm PF, Kampalath B *et al.* Colon biopsies for evaluation of acute graft-versus-host disease (A-GVHD) in allogeneic bone marrow transplant patients. *BMC Gastroenterol* 2003; 3: 5.
54. Stelljes M, Hermann S, Albring J, Köhler G, Löffler M, Franzius C *et al.* Clinical molecular imaging in intestinal graft-versus-host disease: mapping of disease activity, prediction, and monitoring of treatment efficiency by positron emission tomography. *Blood* 2008; 111: 2909–2918.
55. Katzberg RW, Haller C. Contrast-induced nephrotoxicity: clinical landscape. *Kidney Int Suppl* 2006; 69: S3–S7.