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● *Original Contribution*

SPLANCHNIC HEMODYNAMICS AND INTESTINAL VASCULARITY IN CROHN'S DISEASE: AN *IN VIVO* EVALUATION USING DOPPLER AND CONTRAST-ENHANCED ULTRASOUND AND BIOCHEMICAL PARAMETERS

GIOVANNI MACONI,* ANIL K. ASTHANA,† ELENA BOLZACCHINI,* ALESSANDRA DELL'ERA,* FEDERICA FURFARO,* CRISTINA BEZZIO,* VERONICA SALVATORE,‡ and JEANETTE A. M. MAIER§

*Gastrointestinal Unit, Department of Biomedical and Clinical Sciences, "L.Sacco" University Hospital, Milan, Italy; †Department of Gastroenterology, The Alfred Hospital, Melbourne, Australia; ‡Division of Internal Medicine, Azienda Ospedaliero-Universitaria di Bologna Policlinico Sant'Orsola-Malpighi, Bologna, Italy; and §General Pathology, Department of Biomedical and Clinical Sciences, "L.Sacco" University Hospital, Milan, Italy

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Abstract—Crohn's disease (CD) is characterized by inflammation and angiogenesis of affected bowel. We evaluated the correlation among vascularity of intestinal wall in CD, splanchnic hemodynamics, clinical activity and biochemical parameters of inflammation and angiogenesis. Sixteen patients with ileal CD and 10 healthy controls were investigated by means of Doppler ultrasound of the superior mesenteric artery and color Doppler and contrast-enhanced ultrasound of the ileal wall. In parallel, serum levels of vascular endothelial growth factor, tumor necrosis factor-α (TNF-α) and nitric oxide, before and 30 min after a standard meal, were evaluated. In CD patients, there was a significant post-prandial reduction in the resistance index and pulsatility index of the superior mesenteric artery, associated with increased levels of NO and decreased amounts of TNF-α. A correlation was observed between vascular endothelial growth factor and contrast-enhanced ultrasound parameters of intestinal wall vascularity ($r = 0.63–0.71, p < 0.05$) and between these parameters and superior mesenteric artery blood flow after fasting (resistance and pulsatility indexes: $r = -0.64$ and $-0.72, p < 0.05$). Our results revealed a post-prandial increase in nitric oxide and decrease in TNF-α in CD patients *in vivo*. They also confirm the role of vascular endothelial growth factor in angiogenesis and in pathologic vascular remodeling of CD and its effect on splanchnic blood flow. (E-mail: giovanni.maconi@unimi.it) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Crohn's disease, Angiogenesis, Ultrasound, Splanchnic hemodynamics, Contrast-enhanced ultrasound, Vascular endothelial growth factor.**

INTRODUCTION

Crohn's disease (CD) is a chronic disease characterized by persistent inflammation of the intestinal wall and impaired healing of mucosal ulcerations, which is maintained by chronic inflammation and angiogenesis. Angiogenesis is a process of fundamental importance in many physiologic conditions such as tissue growth and wound healing; it is also implicated in many pathologic processes, one of which is CD (Carmeliet 2003). Angiogenesis driven by intestinal inflammation is self-sustaining *via* various inflammatory mechanisms such as enhanced recruitment of inflammatory cells, increased delivery of nutrients and activation of endothelium; this, in turn, contributes to the local production of cytokines, chemokines and matrix metalloproteinases (Deban et al. 2008).

The cellular and molecular mechanisms leading to angiogenesis have been intensively investigated and partially unveiled. Both mechanical stimuli, such as muscle contraction and shear stress, and chemical stimulation *via* angiogenic mediators, such as growth factors (fibroblast growth factor, vascular endothelial growth factor [VEGF], platelet-derived growth factor), can influence blood flow and angiogenesis. As far as the role of muscle contraction is concerned, this may stimulate the production of nitric oxide (NO), resulting in vasodilation (Carmeliet 2003; Hulten et al. 1977). On the other hand, it has been found that NO downregulates human intestinal microvascular endothelial cell activation and leukocyte adhesion; inhibition of inducible nitric oxide

Address correspondence to: Giovanni Maconi, Gastroenterology Unit, Department of Biomedical and Clinical Sciences, Luigi Sacco University Hospital, Via Giovanni Battista Grassi, 74, 20157 Milan, Italy. E-mail: giovanni.maconi@unimi.it

synthetase, a well-known feature of chronically inflamed microvascular endothelial cells, leads to enhanced leukocyte binding and intravascular thrombosis, thereby contributing to angiogenesis and chronic destructive inflammation in inflammatory bowel diseases (Binion et al. 1998; Boughton-Smith 1994; Cromer et al. 2011; Kolios et al. 2004; Sessa 2009).

Most of this evidence derives from studies performed *in vitro* or in experimental animal models. To date, contrast enhanced ultrasound (CEUS) is used to quantitatively assess both tissue or tumor vascularity and the function of microvasculature because it allows real-time and non-invasive assessment of intestinal microvasculature. CEUS is also useful in investigating the response of the tissues to specific drugs, such as anti-angiogenetic drugs (Cosgrove and Lassau 2009; Xia et al. 2011; Yoshida et al. 2011). This technique visualizes only the vascular bed of the intestinal wall (Fleisher 2000; Lassau 2001) and not the interstitial phase, which is usually detected by magnetic resonance imaging and computed tomography, because sulfur hexafluoride (SonoVue), currently the most frequently used ultrasound contrast agent, is a purely blood pool agent, different from computed tomography and magnetic resonance imaging contrast agents. This is relevant as the interstitial space is dependent on microvessel permeability and is markedly increased by disease activity and chronicity.

Moreover, the use of pulsed Doppler ultrasound and color Doppler analysis allows the assessment of splanchnic hemodynamics, thus providing quantitative parameters of blood flow supply to the bowel in the superior mesenteric artery and arterioles within the bowel wall. These parameters correlate with pathologic bowel wall thickening, extension of the disease and, to a lesser degree, the clinical activity of CD (Byrne et al. 2001; Karoui et al. 2010; Maconi et al. 1998; van Oostayen et al. 1994). However, in CD, the relationship between the mechanisms involved in angiogenesis, intestinal vascularity and functional changes in splanchnic hemodynamics remains to be elucidated.

The aim of this study was to assess whether parameters of vascularity of diseased segments of intestinal wall correlate with biochemical markers of disease activity and, particularly, with biochemical parameters of inflammation and angiogenesis.

METHODS

Patients

The study included 16 patients aged between 18 and 60 y who had an established diagnosis of Crohn's disease; 10 healthy volunteers, enrolled from the home medical unit medical and nursing staff, were matched for age and sex. Exclusion criteria were: Crohn's disease localized in the colon and/or in the upper gastrointestinal tract, peri-anal Crohn's disease, current treatment with immunosuppressants or biologics (*e.g.*, infliximab, adalimumab, azathioprine or methotrexate), concomitant diseases (*e.g.*, diabetes mellitus, malignant tumors, ischemic heart and cerebral diseases) and a history of multiple intestinal resections.

Study design

The institutional review board approved the study, and all patients gave their written formal informed consent to participate; the study was conducted according to the principles of the Declaration of Helsinki. Demographic and clinical data were collected using a standard questionnaire; CD behavior and clinical disease activity were assessed with the Montreal classification (Silverberg et al. 2005). The Crohn's Disease Activity Index (Best et al. 1976) and endoscopic disease activity (erosions or ulcers) were documented at the participant's latest endoscopic assessment (<6 mo). All patients underwent an intestinal ultrasound scan (IUS) for assessment of disease location and activity and degree of thickening of the diseased bowel walls.

In addition, all patients underwent the following examinations, before and 30 min after the intake of 200 mL of caloric meal (300 kCal):

1. Doppler ultrasound (US) of the splanchnic vessels included assessment of the (i) resistance index (RI) and pulsatility index (PI) of the superior mesenteric artery (SMA); and the (ii) mean velocity, diameter and total blood flow of the portal vein (PV).
2. Pulsed Doppler and color Doppler US of the diseased bowel segment at the level of maximum bowel wall thickness were used to determine (i) the RI of arterioles detected with color Doppler and (ii) the vascularity of the bowel wall, which was assessed using various parameters (discussed later) obtained with CEUS.
3. Serologic samples were obtained to determine inflammatory indexes (C-reactive protein, tumor necrosis factor-α [TNF-α]), angiogenesis markers (VEGF) and endothelial function markers (NO).

Sonographic and Doppler examinations

All sonographic examinations were performed by the same operator (G.M.), who was blinded to the biochemical and clinical data of the patients but was aware of the diagnosis (Crohn's disease or healthy control) and any relevant history of previous surgeries.

Intestinal US and Doppler US were performed in the morning, after overnight fasting and after the standard caloric meal. The following parameters were collected: maximum bowel wall thickness; extension of thickened bowel wall segments; echo patterns of bowel wall segments; presence of intestinal complications such as

strictures, fistulas and abscesses, as previously described (Maconi et al. 2006). All of these examinations were performed using a real-time scanner (Hitachi Logos Hi-Vision C, Tokyo, Japan) and a high-resolution microconvex probe (4–8 MHz).

Doppler US assessment of the SMA and PV was performed in the supine position after 15 min of rest, with the patient's breath held in expiration and using the same equipment—a 3.5-MHz convex transducer with a pulsed Doppler device operating at a frequency of 2.5 MHz—as previously described (Norris et al. 1984). In particular, the time-averaged spatial mean velocity, V_{mean} (cm/s), and anteroposterior (AP) diameter of the PV and SMA, as well as the RI and PI of the SMA, were measured. V_{mean} was calculated directly by the instrument software by integrating the area under each individual velocity waveform. Portal and mesenteric flow volumes were calculated from the V_{mean} and AP diameter of the vessels according to the formula $Q = V_{mean} \times A$, where Q = volume flow, V_{mean} = time-averaged mean velocity of blood flow in the vessel and A = cross-sectional area of the vessel. Because the instrument software provided time-averaged maximal velocity, flow volume was calculated using the spatial-average, time-average flow velocity obtained by multiplying maximal velocity by 0.57, as previously reported in literature (Piscaglia et al. 1998). The RI and PI of the SMA were calculated as RI = $(S - D)/S$ and PI = $(S - D)/V_{mean}$, where S = peak systolic velocity and D = end-peak diastolic velocity.

Color Doppler assessment of thickened bowel segments was performed using a linear high-resolution probe (5–7.5 MHz), with a specific pulse repetition frequency of 400–800 MHz and gain of 6–18, detect intra-parietal slow blood flow. Once the intraparietal blood vessels were identified, spectral analysis of the blood flow within the vessels was performed using an adequate sample volume (3–5 mm^3), and in the event of arterial flow, the RI was calculated.

The RI and PI are semi-quantitative indexes related to the impedance of the arterial bed, which includes resistance, compliance and congestion of the vascular bed (Maconi et al. 1998; Barbara 1990). Furthermore, RI is independent of the angle of insonation. Three consecutive evaluations were carried out for each parameter of the splanchnic vessels (V_{mean}, AP diameter, RI and PI) and intra-parietal blood flow (RI), and the mean value was calculated.

Contrast-enhanced ultrasound

All patients underwent real-time CEUS performed with an Esaote MyLab 70 Gold (Esaote, Genoa, Italy) with a multiband high-frequency transducer (3–11 MHz). This transducer transmits at 2.5 MHz and receives at 5 MHz when used with the contrast-specific non-linear harmonic software, thus optimizing real-time detection of harmonic contrast responses with the following general settings: color map 2.0, dynamic range 60, low acoustic power setting, at 40 kPa pressure (Fig. 1), expressing a mechanical index of 0.7. The gain was also adjusted to reduce background, and one focus was kept below the level of the target bowel wall. All technical settings, including gain and acoustic power (kPa), were not modified between the investigations performed before and after a meal for each patient. Minimal variations were used in only two patients.

CEUS was performed after injection into an arm vein of a 2.4-mL bolus of sulfur hexafluoride-filled microbubbles (SonoVue) followed by 10 mL of saline solution. Sixty-second cine-clips of the same bowel loop assessed by unenhanced US, Doppler US and CEUS scan were stored on digital devices (Fig. 1).

Quantitative analysis of the vascularity of the intestinal wall was performed off-line using a dedicated software program (Q-contrast, distributed by Bracco) on the wide region of interest that encompassed the selected thickened bowel wall in longitudinal view, that is, along the mesenteric side as previously reported (Migaleddu et al. 2011). In brief, this software quantifies the contrast enhancement from a time sequence of frames and creates a chromatic map that allows evaluation of the vascularity of the bowel segment encompassed by the wide region of interest. A virtual color image of the bowel wall, ranging from red (maximum signal intensity) to blue (minimum signal intensity), was obtained.

In this segment of bowel wall, three different regions of interest (ROI) with the highest echo signal intensity after microbubble injection were considered. Time–intensity curves from these ROIs were obtained, and various kinetic parameters calculated. The median of each kinetic parameter was used as the vascularity parameter. The measurements obtained from parametrized data included: (i) slope of the first ascending tract of the curve (*i.e.*, refilling time); (ii) time required for refilling 63% of the vascular bed at its maximum value, the time related to the wash-in time and peak enhancement (signal intensity); and (iii) area under the enhancement curve (regional blood flow volume).

These parameters were assessed on the same bowel loop before and after meals. The consistency in scan and image of the same intestinal tract before and after a meal was possible because specific, easily recognizable bowel segments were selected. The same loop segment was always evaluated (anterior anti-mesenteric site) by using previous scans to recognize the exact sites. The potential effects of overlying tissues and variation in depth of the ROIs were not considered in the study, because the short interval between the two study time points and the relatively small meal volume between the two assessments in each patient were not expected to modify them.

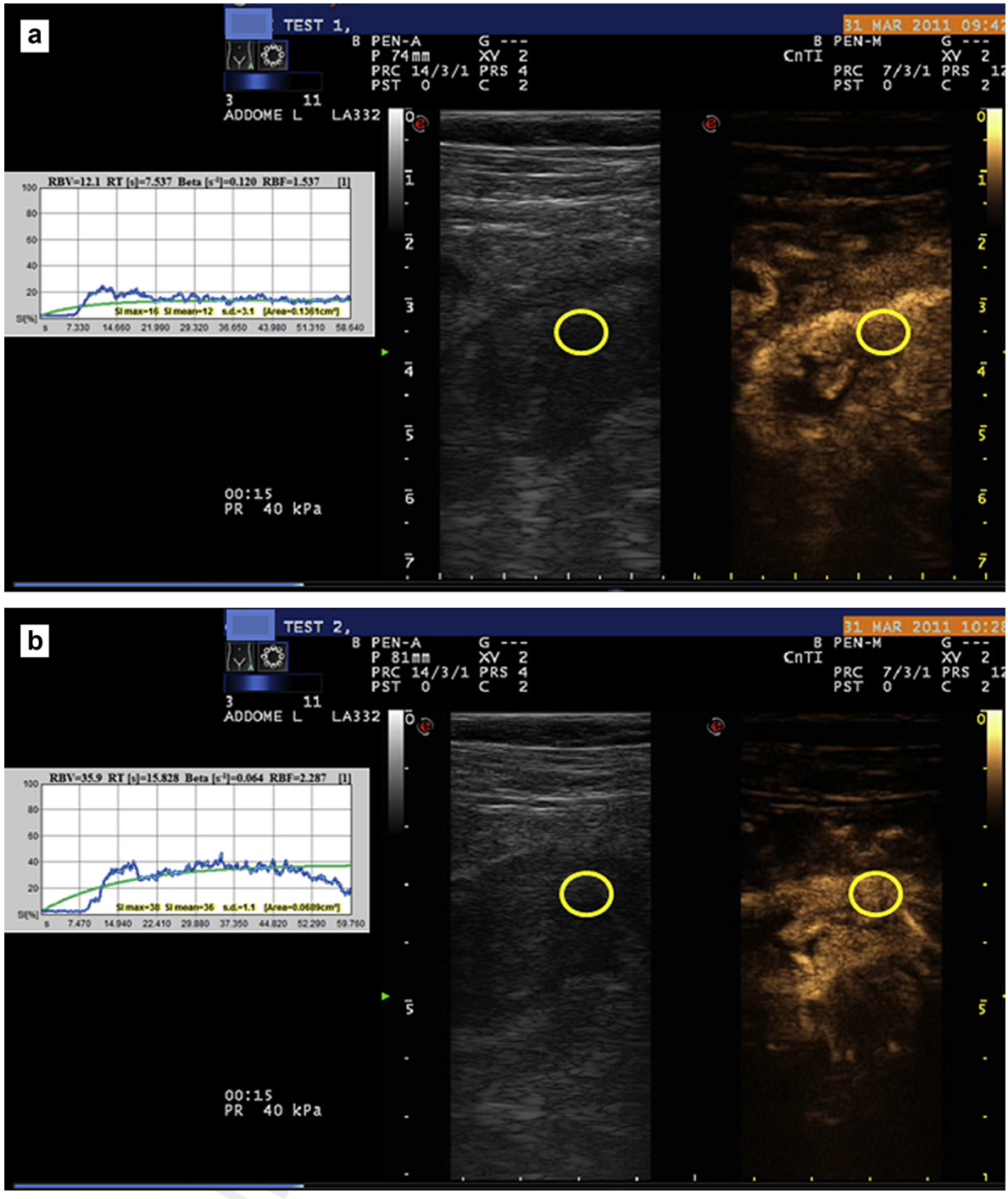

Fig. 1. B-Mode and contrast-enhanced ultrasound scan (right) revealing enhancement of the wall of the terminal ileum, and echo signal intensity-versus-time curve (left) within a region of interest, before (a) and 30 min after (b) the standard meal.

Biochemical parameters

For each patient, C-reactive protein and total blood cell count were assessed. Moreover, VEGF-A and TNF-α levels were determined with the commercially Quantikine Immunoassay (R&D Systems, Minneapolis, MN, USA) enzyme-linked immunosorbent assay kit according to the manufacturer's instruction.

Nitric acid synthase activity was determined using the Griess assay for nitrite quantification on 50 μL of serum. In this method, nitrite is first treated with a diazotizing reagent in acidic medium to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, *N*-naphthyl-ethylenediamine, to form a stable azo compound. The intense purple color of

the product allows the nitrite assay to be used with a high level of sensitivity and can thus be used to measure nitrite concentration as low as ~0.5 μM. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample. In this assay, we used the sequential method, which seems to give the highest yield of chromophore and, therefore, is the most sensitive way to perform the Griess reaction assay (Guevara et al. 1998).

Standard liquid meal

The standard liquid meal consisted of 200 mL of aqueous solution (70% water) containing a mixture of protein (11.2 g), fat (10 g), carbohydrates (42 g), vitamins and mineral salts of 300 kcal (1280 kJ); the osmolarity of this solution was 445 mOsm/L (Resource Energy, Nestlè Italia, Milan, Italy).

Statistical analysis

Each pre-meal measurement was compared with the corresponding post-prandial measurement using Student's *t*-test for paired data. Basal and post-prandial variations in biochemical and sonograhic variables were compared with clinical, endoscopic and biochemical indexes using the Spearman rank test correlation. Doppler US and vascularity parameters assessed before and after each meal were compared using the Wilcoxon test.

RESULTS

Patients

Demographic data and clinical features of the study patients included are summarized in Table 1.

At sonography, the bowel wall had an average thickness of 7.3 mm and a stratified echo pattern in all cases. Six patients had sonographic features suggestive of an ileal stricture, and five patients had an entero-enteric fistula (Table 1). The healthy controls were similar with respect to sex (four males), age (mean age 34 y) and smoking habit (three smokers); for all healthy controls, the terminal ileum had a normal sonographic appearance.

Splanchnic hemodynamics and intestinal blood flow

Crohn's disease patients exhibited a tendency toward hyperdynamic splanchnic blood flow compared with healthy controls, but only the end-diastolic velocity was significantly higher (Table 2).

In the post-prandial period, both patients and healthy controls had a significant and comparable increase in splanchnic blood flow, namely, comparable and significant reductions in the RI and PI of the SMA, along with significant increases in total blood flow and portal blood flow volume.

Table 1. Demographic and clinical characteristics of patients

Characteristic	Mean (SD)	n (%)
Age (y)	39 (13.8)	
Age at diagnosis (y)	31 (7.8)	
Disease length (y)	10	
Sex (males)		8 (50)
Active smokers		7 (43)
Previous surgical operation for Crohn's disease		4 (25)
Disease extension (cm)	17 (11)	
Maximum bowel wall thickness (mm)	7.3 (1.02)	
Presence of stenosis		6 (37)
Disease behavior (Montreal classification)		
Inflammatory (B1)		5 (31)
Stricturing (B2)		6 (38)
Penetrating (B3)		5 (31)
Current therapy		
None		8 (50)
Mesalazine		5 (31)
Budesonide (<6 mg d)		3 (19)
CDAI		
Patients with active disease (CDAI >150)	82 (72.0)	2 (12)
C-Reactive protein, median (range)	5.5 (0.6–18.5)	

CDAI = clinical disease activity index.

Observation of the vascularity of the intestinal wall, by means of color Doppler with spectral analysis, was feasible in the basal and post-prandial periods in CD patients only. The RI of intestinal flow significantly decreased in the post-prandial period (0.65 vs. 0.60, $p < 0.05$) (Table 2).

Contrast-enhanced ultrasound

Contrast-enhanced ultrasound was performed in all patients. However, secondary to artifacts from bowel peristaltic movements and overlying gas, bowel vascularity indexes in both the basal and post-prandial periods were accurately assessed in 13 patients and 4 healthy controls. All of the indexes assessed exhibited wide variability, but without significant differences between patients and controls; this was the case in both the basal and post-prandial periods as well as between the fasting and feeding states (Table 2).

Biochemical assessment

Basal serum levels of VEGF, TNF-α and NO in CD patients did not exhibit significant differences compared with those of healthy controls (Table 2). On the contrary, in the post-prandial period, healthy controls did not have any significant changes, whereas CD patients had a significant increase in NO levels and a significant decrease in TNF-α levels (Table 2).

Clinical features and splanchnic blood flow in Crohn's disease

No significant correlation was observed between splanchnic blood flow parameters (RI and PI of

Table 2. Characteristics of the splanchnic and intestinal vascularity and biochemical parameters in fasting and post-prandial period in healthy controls and CD patients

	Healthy controls (n = 10)			CD patients (n = 16)		
	Pre	Post	*p*	Pre	Post	*p*
Superior mesenteric artery*						
Resistance index	0.845 (0.024)	0.78 (0.05)	0.010	0.853 (0.039)	0.78 (0.04)	0.001
Pulsatility index	2.34 (0.31)	1.87 (0.28)	0.003	2.75 (0.65)	1.39 (0.39)	0.001
Peak systolic velocity (cm/s)	168.9 (33.25)	237.0 (63.63)	0.003	200.6 (46.93)	249.8 (45.2)	0.005
End-diastolic velocity (cm/s)	25.34 (6.76)	52.43 (10.98)	0.003	29.0 (9.21)	55.58 (13.4)	0.001
Portal vein*						
Mean of maximum velocities (cm/s)	26.73 (5.14)	32.67 (3.60)	0.012	26.36 (2.89)	34.17 (6.62)	<0.001
Diameter (mm)	12.52 (1.16)	13.32 (1.90)	0.154	13.34 (1.30)	13.86 (1.26)	0.066
Flow volume (mL/min)†	1881 (499)	2691 (1076)	0.035	2107 (404)	2962 (775)	<0.001
Pulsed Doppler of intestinal wall‡						
Resistance index	NA	NA		0.644 (0.058)	0.607 (0.095)	0.017
Bowel wall perfusion§						
Regional blood flow volume (AU)	15.75 (5.06)	14.1 (1.96)	0.461	12.53 (6.1)	17.3 (11.7)	0.359
Refilling time (s)	19.9 (10.5)	10.5 (4.7)	0.715	14.62 (8.3)	14.9 (11.7)	0.213
Peak enhancement (%)	21.5 (6.4)	24.25 (9.91)	0.786	20.25 (5.49)	22.25 (10.1)	0.695
Biochemical parameters						
Nitric oxide (μmol/L)	21.2 (5.8)	21.8 (2.9)	0.576	17.3 (1.3)	21 (2.2)	0.002
VEGF (ng/mL)	141.3 (64.5)	132.8 (64.3)	0.208	209.5 (145.1)	224.7 (5.84)	0.727
TNF-α (U/mL)	79.2 (15.0)	73.7 (19.7)	0.674	80.3 (14.0)	64.4 (12.6)	0.007

AU = arbitrary units; CD = Crohn's disease; TNF-α = tumor necrosis factor α; VEGF = vascular endothelial growth factor.
* Assessable before and after meal in 8 healthy controls and 15 CD patients.
† Portal blood flow volume was calculated from portal vein sectional area and mean velocity, obtained from maximum velocity multiplied by 0.57 (Piscaglia et al. 1998).
‡ N.A. not assessable in healthy controls because of the thin walls of the bowel, very small diameters of vessels and motion artifacts, and accurately assessable before and after meal in 12 patients.
§ Not assessable before and after meal in 4 healthy controls and 12 CD patients.

SMA and PV blood flow) and the main clinical features of CD patients, except for significant inverse correlations between the RI of the SMA and duration of CD ($r = -0.596$, $p < 0.05$) and between PV blood flow and intestinal strictures ($r = 0.592$, $p < 0.05$). In addition, the indexes of parietal blood flow, along with serum levels of NO, VEGF and TNF-α, were not significantly correlated with duration, activity, extension or complications of CD or previous surgery (Table 3).

Splanchnic hemodynamics, intestinal blood flow and biochemical indexes

Intestinal vascularity parameters obtained by CEUS in the fasting state were significantly correlated with basal serum levels of VEGF but not with serum basal levels of NO and TNF-α. On the contrary, post-prandial serum levels of VEGF, NO and TNF-α were not significantly correlated with intestinal vascularity indexes (Table 4).

Vascularity indexes of the intestinal wall assessed with CEUS were significantly correlated with PV blood flow and the RI and PI of the SMA, but only in the fasting state. In the post-prandial period, we did not observe any significant correlations among CEUS vascularity indexes of the intestinal wall and SMA or PV parameters of blood flow (Table 5).

DISCUSSION

This study indicates that splanchnic blood flow correlates with vascularity of the intestinal wall, which, in

Table 3. Correlations (*r*, Spearman rank test) among the clinical parameters and fasting splanchnic blood flow, bowel wall perfusion and biochemical parameters

Parameter	RI-SMA	PI-SMA	PV-F	RBV	RT	PE	NO	VEGF	TNF-α
Duration of disease	−0.596*	−0.398	−0.320	0.164	0.369	0.058	0.120	0.321	−0.277
Disease activity	0.213	−0.021	0.211	0.234	0.211	0.494	−0.085	−0.127	0.148
Disease extension	0.083	−0.186	0.165	0.068	0.194	−0.377	−0.227	0.041	−0.145
Previous surgery	−0.493†	−0.201	−0.312	−0.366	−0.330	−0.531*	−0.220	−0.089	−0.245
Strictures	0.021	−0.190	0.592*	0.153	0.180	−0.040	0.148	0.380	0.169

CD = Crohn's disease; PE = peak enhancement; PI-SMA = pulsatility index of SMA; RBV = regional blood flow volume; RI-SMA = resistance index of SMA; RT = refilling time; TNF-α = tumor necrosis factor α; VEGF = vascular endothelial growth factor.
* $p < 0.05$.

Table 4. Correlation (*r*, Spearman rank test) between biochemical parameters and intestinal vascularity before and after a meal in patients with Crohn's disease

	Before meal			After meal		
	NO	VEGF	TNF-α	NO	VEGF	TNF-α
Pulsed Doppler of intestinal wall						
Resistance index	−0.150	0.391	−0.320	−0.220	−0.246	0.164
CEUS of intestinal wall						
Regional blood flow volume	0.170	0.630*	−0.280	−0.406	−0.067	0.067
Refilling time	0.180	0.612†	−0.330	−0.210	−0.164	0.139
Peak enhancement	0.180	0.710‡	−0.372	−0.406	−0.140	−0.370

CEUS = contrast-enhanced ultrasound; TNF-α = tumor necrosis factor α; VEGF = vascular endothelial growth factor.
* $p = 0.047$.
† $p = 0.060$.
‡ $p = 0.013$.

turn, correlates with angiogenesis. It also indicates that only in CD patients does NO increase, whereas TNF-α decreases after a standard meal.

To date, a correlation between angiogenesis, bowel vascularity and splanchnic hemodynamics has been hypothesized, but not fully elucidated in humans, and the few studies available revealed a correlation between some of these parameters, essentially angiogenesis and bowel vascularity, in patients with active CD (Byrne et al. 2001; Di Sabatino et al. 2004; Ripolles et al. 2008; van Oostayen et al. 1994). In particular, molecular experimental evidence implicated intestinal microvascular remodeling or angiogenesis phenomena in the pathogenesis of CD (Pousa et al. 2008). However, this issue has been poorly investigated *in vivo* in humans so far.

Our results indicate for the first time that in patients with ileal CD in clinical remission, bowel vascularity correlates with splanchnic hemodynamics, but not with biochemical parameters of CD, such as TNF-α. This finding could explain why in some CD patients successfully treated with anti-TNF-α agents, ileal bowel wall thickening and vascularity may persist despite the clinical remission. Moreover, because inflammation may be favored and maintained by pathologic angiogenesis, our results reinforce the hypothesis of angiogenesis as a potential target of therapies in CD. Although statistical significance was not achieved, our results indicate that VEGF levels tend to be higher in CD patients than in healthy controls.

In line with current literature (Giovagnorio 1999), we found increased post-prandial blood flow in the SMA and PV of both patients and healthy controls and a significant fall in arteriolar resistance in patients (0.65 vs. 0.60, $p < 0.05$). However, the capillary vascularity of the bowel wall did not differ significantly between CD patients and healthy controls, both at baseline and in the post-prandial period. This finding is likely a result of the high variability in measurements of vascularity parameters and the heterogeneity of the features of the patients. We hypothesize that resistance may have decreased because of the increased amounts of NO in CD patients after the meal. Indeed, although serum levels of VEGF, TNF-α and NO of CD patients at baseline did not significantly differ from those of healthy controls, in the post-prandial period CD patients had a significant increase in NO and a significant decrease in TNF-α. Although increased fasting serum levels of NO and TNF-α are well-known features of CD (Kolios et al. 1998; Plevy et al. 1997), no data are available at present regarding post-prandial levels. The increase in NO levels could be interpreted as the amplification of a physiologic effect, as it may result from muscular contraction, which occurs during the post-prandial period (Binion et al. 1998; Boughton-Smith 1994; Cromer et al. 2011; Kolios et al. 2004; Sessa 2009). The decrease in TNF-α levels is unexpected and unexplained because of its crucial role in the

Table 5. Correlation (*r*, Spearman rank test) among perfusion parameters of the intestinal wall and splanchnic vascularity in patients with Crohn's disease before and after a standard meal

	Before meal			After meal		
	RI-SMA	PI-SMA	PV-F	RI-SMA	PI-SMA	PV-F
Pulsed Doppler US of intestinal wall						
Resistance index	−0.349	−0.443	0.135	0.242	0.076	0.096
Perfusion parameters of intestinal wall						
Regional blood flow volume	−0.426	−0.484	0.793*	−0.255	−0.222	−0.382
Refilling time	−0.639‡	−0.724†	0.691†	−0.173	−0.210	−0.182
Peak enhancement	−0.461	−0.545	0.660†	−0.218	−0.221	−0.358

RI-SMA = resistance index of superior mesenteric artery; PI-SMA = pulsatility index of superior mesenteric artery; PV-F = portal vein flow; US = ultrasound.
* $p = 0.004$.
† $p < 0.012$.
‡ $p = 0.034$.

pathogenesis of CD. Indeed, different anti-TNFα agents have been approved for the treatment of CD patients. We hypothesize that the decreased serum levels of TNF-α may have been triggered by some components of the nutrient drink used as a meal test, but this issue requires further investigation.

The main finding of our study is the significant correlation between vascularity parameters of the ileal wall and VEGF serum levels detected in the fasting state. These findings, which are in keeping with those of Di Sabatino et al. (2004) (although obtained with different methodology and in CD patients with active disease), confirm the role of VEGF in angiogenesis and vascular remodeling of CD, also in patients in clinical remission. The link between inflammation and angiogenesis suggests that angiogenesis could also be a potential target of therapies for CD. The relationship between parietal blood flow and VEGF has been observed only in the fasting state. This may be attributed to the fact that in post-prandial period, other factors may affect vascularity of the bowel wall (*e.g.*, gastrointestinal hormones, enteric nervous system). NO is included among the factors that may play a role, as suggested by the results of our study.

We also observed a correlation between vascularity indexes of the ileal wall and increased blood flow in the PV and decreased vascular resistance (RI and PI) of the SMA; this was the case in the fasting state only. These findings confirm that in CD, the diseased bowel wall is the main culprit in impaired splanchnic blood flow (Maconi et al. 1998) and its parietal vascularity plays a relevant role.

We acknowledge that this study may be limited by the small number of patients included, the high variability of some measurements of intestinal blood flow and the failure to obtain some parameters of bowel vascularity in healthy controls. Furthermore, the depth and overlying tissues of the regions of interest where we assessed bowel vascularity *in vivo* account for this variability and may have somewhat decreased accuracy in assessing directly the post-prandial intestinal blood flow at the exact site of the fasting examination. In particular, in healthy controls, assessment of vascularity with CEUS in the same intestinal segments before and after a meal was possible in only 4 of 10 individuals.

Although limited by both the small number of subjects included and the inconsequential technical limitations, the findings of this study, which first assessed simultaneously both splanchnic/intestinal blood flow parameters and biochemical markers of vascularity in CD, may explain most of the recent published data on vascularization of the bowel in inflammatory bowel diseases, and confirm that CEUS results may be useful in assessing angiogenesis and hence CD activity.

In conclusion, our study indicates that intestinal flow is related to angiogenesis and affects splanchnic hemodynamics.

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REFERENCES

Barbara L. The value of Doppler US in the study of hepatic haemodynamics. Consensus conference. J Hepatol 1990;10:353–355.

Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index: National Cooperative Crohn's Disease Study. Gastroenterology 1976;70:439–444.

Binion DG, Fu S, Ramanujam KS, Chai YC, Dweik RA, Drazba JA, Wade JG, Ziats NP, Erzurum SC, Wilson KT. iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion. Am J Physiol 1998;275:G592–G603.

Boughton-Smith NK. Pathological and therapeutic implications for nitric oxide in inflammatory bowel disease. J R Soc Med 1994;87:312–314.

Byrne MF, Farrell MA, Abass SL, Fitzgerald A, Varghese JC, Thornton F, Murray FE, Lee MJ. Assessment of Crohn's disease activity by Doppler sonography of the superior mesenteric artery, clinical evaluation and the Crohn's disease activity index: A prospective study. Clin Radiol 2001;56:973–978.

Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653–660.

Cosgrove D, Lassau N. [Assessment of tumour angiogenesis using contrast-enhanced ultrasound]. J Radiol 2009;90:156–164.

Cromer WE, Mathis JM, Granger DN, Chaitanya GV, Alexander JS. Role of the endothelium in inflammatory bowel diseases. World J Gastroenterol 2011;17:578–593.

Deban L, Correale C, Vetrano S, Malesci A, Danese S. Multiple pathogenic roles of microvasculature in inflammatory bowel disease: A jack of all trades. Am J Pathol 2008;172:1457–1466.

Di Sabatino A, Ciccocioppo R, Armellini E, Morera R, Ricevuti L, Cazzola P, Fulle I, Corazza GR. Serum bFGF and VEGF correlate respectively with bowel wall thickness and intramural blood flow in Crohn's disease. Inflamm Bowel Dis 2004;10:573–577.

Fleisher AC. Sonographic depiction of tumor vascularity and flow: from *in vivo* models to clinical applications. J Ultrasound Med 2000;19:55–61.

Giovagnorio F. Doppler ultrasonography of the upper mesenteric artery in chronic intestinal inflammation. Radiol Med 1999;98:43–47.

Guevara I, Iwanejko J, Dembinska-Kiec A, Pankiewicz J, Wanat A, Anna P, Gołabek I, Bartuś S, Malczewska-Malec M, Szczudlik A. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta 1998;274:177–188.

Hulten L, Lindhagen J, Lundgren O, Fasth S, Ahren C. Regional intestinal blood flow in ulcerative colitis and Crohn's disease. Gastroenterology 1977;72:388–396.

Karoui S, Nouira K, Serghini M, Ben Mustapha N, Boubaker J, Menif E, Filali A. Assessment of activity of Crohn's disease by Doppler sonography of superior mesenteric artery flow. J Crohns Colitis 2010;4:334–340.

Kolios G, Rooney N, Murphy CT, Robertson DAF, Westwick J. Expression of inducible nitric oxide synthase activity in human colon epithelial cells: Modulation by T-lymphocyte derived cytokines. Gut 1998;43:56–63.

Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: A universal messenger in an unsolved puzzle. Immunology 2004;113:427–437.

Lassau N. Evaluation of contrast-enhanced color Doppler ultrasound for the quantification of angiogenesis *in vivo*. Invest Radiol 2001;36:50–55.

Maconi G, Parente F, Bollani S, Imbesi V, Ardizzone S, Russo A, Bianchi Porro G. Factors affecting splanchnic haemodynamics in

Crohn's disease: A prospective controlled study using Doppler ultrasound. Gut 1998;43:645–650.

Maconi G, Radice E, Greco S, Bianchi Porro G. Bowel ultrasound in Crohn's disease. Best Pract Res Clin Gastroenterol 2006;20:93–112.

Migaleddu V, Quaia E, Scanu D, Carla S, Bertolotto M, Campisi G, Sirigu D, Virgilio G. Inflammatory activity in Crohn's disease: CE-US. Abdom Imaging 2011;36:142–148.

Norris CS, Pfeiffer JS, Rittgers SE, Barnes RW. Noninvasive evaluation of renal artery stenosis and renovascular resistance: Experimental and clinical studies. J Vasc Surg 1984;1:192–201.

Piscaglia F, Gaiani S, Gramantieri L, Zironi G, Siringo S, Bolondi L. Superior mesenteric artery impedance in chronic liver diseases: Relationship with disease severity and portal circulation. Am J Gastroenterol 1998;93:1925–1930.

Plevy SE, Landers CJ, Prehn J, Carramanzana NM, Deem RL, Shealy D, Targan SR. A role of TNF-α and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol 1997;159:6276–6282.

Pousa ID, Maté J, Gisbert JP. Angiogenesis in inflammatory bowel disease. Eur J Clin Invest 2008;38:73–81.

Ripollés T, Martínez MJ, Barrachina MM. Crohn's disease and color Doppler sonography: Response to treatment and its relationship with long-term prognosis. J Clin Ultrasound 2008;36:267–272.

Sessa WC. Molecular control of blood flow and angiogenesis: Role of nitric oxide. J Thromb Haemost 2009;7(Suppl 1):35–37.

Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV Jr, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005;19(Suppl A):5A–36A.

van Oostayen JA, Wasser MN, van Hogezand PA, Griffioen G, de Roos A. Activity of Crohn disease assessed by measurement of superior mesenteric artery flow with Doppler US. Radiology 1994; 193:551–554.

Xia Y, Jiang YX, Dai Q, Xiao Y, Lv K, Wang L. Contrast-enhanced ultrasound of hepatocellular carcinoma: Correlation of washout time and angiogenesis. Clin Hemorheol Microcirc 2011;48: 265–273.

Yoshida K, Hirokawa T, Moriyasu F, Liu L, Liu GJ, Yamada M, Imai Y. Arterial-phase contrast-enhanced ultrasonography for evaluating anti-angiogenesis treatment: A pilot study. World J Gastroenterol 2011;17:1045–1050.