# Boiling Histotripsy as a Non-Invasive Non-Thermal Approach for Treatment of Human Colon Cancer Tumors

## An *Ex Vivo* Proof-of-Concept

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*Received June 3, 2025, from the Physics Faculty, Lomonosov Moscow State University, Moscow, Russia (E.P., S.T., D.C., P.P., M.K., L.P., A.K., V.K.); Lomonosov Moscow State University Clinic, Medical Scientific and Educational Institute, Moscow, Russia (A.K., N.D., P.M.); Diagnostic Ultrasound Division, Russian Medical Academy of Continuous Professional Education, Moscow, Russia (A.K.); Department of Fundamental Pulmonology, Pulmonology Scientific Research Institute, Moscow, Russia (A.C.); and Faculty of Fundamental Medicine, Lomonosov Moscow State University, Moscow, Russia (S.B.). Manuscript accepted for publication November 3, 2025.*

*This work was performed within the Moscow State University Program of Development (project no. 24-SCH06-12). Ekaterina Ponomarchuk was supported by a student stipend from the Foundation for the Advancement of Theoretical Physics and Mathematics "BASIS" (no. 20-2-10-10-1). Leica SCN 400 slide scanner acquired under the MSU development program was used for digitalization of the histological slides. The authors are also grateful to T.I. Milenkovich for assistance in sample preparation for BH treatment and S.A. Usmanova for preparing histological specimens. The authors declare no conflicts of interest.*

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**Abbreviations**

*BH, boiling histotripsy; CRC, colorectal cancer; H&E, hematoxylin–eosin; HIFU, high-intensity-focused ultrasound; MT, Masson's trichrome; SWE, shear wave elastography; US, ultrasound*

doi:10.1002/jum.70137

***Objectives***—Colorectal cancer (CRC) is the third most common cancer globally and is frequently diagnosed at metastatic stages. As an alternative to the current standard of care, a novel high-intensity-focused ultrasound (HIFU)-based boiling histotripsy (BH) method has the potential to provide an ultrasound (US)-guided approach for non-invasive mechanical fractionation of CRC metastases and enhancement of anti-tumor immune response for inoperable primary CRC.

***Methods***—Young's modulus of the autopsy human sigmoid colon cancer was first measured using shear wave elastography. Volumetric BH lesion was then produced in the cancer sample *ex vivo* using a 2-MHz 9-element annular array (67.5° focusing angle) targeting a 3D-grid (5 × 5 foci across 2 transverse layers with 1-mm spacing) under B-mode US guidance. Each focus received 150 pulses of 1-ms duration (peak positive/negative pressures and shock amplitude $P+/P/A_s = 91/-13/78$ MPa, acoustic power and initial intensity within the pulse $W_0 = 125.5$ W and $I_0 = 2.7$ W/cm$^2$) delivered at 0.1-s intervals (1% duty cycle). The obtained lesion was analyzed *via* B-mode imaging and histologically with hematoxylin–eosin and Masson's trichrome.

***Results***—Young's modulus of the autopsy CRC sample was 38 ± 10 kPa and aligned with clinical values for rectal adenocarcinomas. BH treatment was successfully guided in real-time using B-mode US: the treated area was hyperechoic during BH and hypoechoic post-treatment. Histology confirmed the presence of intact mucinous colon carcinoma outside the BH lesion, and fractionated cells and damaged stroma with disrupted collagen fibers within the lesion.

***Conclusions***—This *ex vivo* pilot study demonstrates the potential of BH for non-invasive non-thermal US-guided treatment for CRC and metastases.

***Key Words***—colorectal cancer; HIFU; histotripsy; local ablation; metastases; non-invasive surgery

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide.[1,2] Early-stage CRC can be resected through polypectomy during colonoscopy, endoscopic mucosal resection or laparoscopically. However, CRC is often asymptomatic at early stages and, therefore, is mostly diagnosed at advanced stages when metastatic.[2] Standard of care for more advanced CRC currently

includes open laparoscopic surgical resection, chemotherapy, radiation therapy, and immunotherapy, all having adverse effects and significantly affecting the patient's quality of life.[3] Ultrasound (US)-enhanced chemotherapy is currently being investigated to increase drug penetration into CRC (eg, doxorubicin and epirubicin).[4–6] As a non-invasive approach for local ablation of CRC, transrectal high-intensity-focused ultrasound (HIFU) ablation is currently undergoing clinical trials to focally heat the tumor through absorption of US energy leading to thermal necrosis of the tumor.[7,8] However, thermal HIFU ablation has some limitations, such as limited treatment accuracy due to heat sink (caused by blood perfusion) and heat diffusion effects restricting or extending the ablated region beyond the focal spot onto surrounding healthy tissues. MRI guidance has been a gold standard to calculate the ablated volume based on the measured temperature versus time curves in each MRI voxel. Such approach has been proven clinically but is more costly and difficult to provide than diagnostic US, which, however, has limited ability to differentiate intact from thermally ablated tissues.[9]

As an alternative to thermal HIFU ablation of CRC and its metastasis, another non-invasive HIFU-based approach, allowing for mechanical, that is, non-thermal, disintegration of tissues, and termed *histotripsy*, is currently being investigated.[10,11] As opposed to relatively long continuous exposures (from seconds to tens of seconds) used in thermal HIFU, histotripsy transducer focuses sequences of very short (from microseconds to several milliseconds) high-amplitude US pulses onto the target tissue. Shock fronts, that is, drastic changes in pressure, form in the pressure waveform at the HIFU focus due to non-linear propagation effects.[12,13] Absorption of the US energy at such shock fronts is significantly higher than that in thermal HIFU protocols since the heat deposition in shock-wave regimes is proportional to the shock amplitude cubed whereas absorption of harmonic wave energy (typical for thermal HIFU) is proportional to US intensity, that is, to the pressure amplitude squared. This feature of shock waves is fundamental for the *boiling histotripsy* (BH) method.[13,14] BH utilizes millisecond-long pulses, within which enhanced tissue heating allows for initiation of very rapid and highly localized boiling of tissue at the focus. The interaction of the remaining shocks of each pulse with the vapor bubbles eventually results in mechanical fractionation, or liquefaction, of tissue into subcellular fragments through acoustic atomization, cavitation, and microstreaming mechanisms.

The boiling and cavitation bubbles that are present during histotripsy exposure act as strong scatterers of US and appear hyperechoic (ie, bright) on diagnostic US. Post-treatment, the liquefied tissue is devoid of scatterers, and, therefore, appears hypoechoic (ie, dark). Thus, BH can be guided in real time *via* conventional diagnostic US instead of MRI thermometry. Further, liquefied content can be easier and faster removed from the body without scarring as compared to thermal HIFU. Moreover, the non-thermal mechanism of histotripsy limits heat diffusion beyond the focal spot and, thus, increases the precision and control of the histotripsy treatment.

Compared to another histotripsy type based on generation of dense cavitation clouds in tissue, which is currently undergoing clinical trials for the treatment of primary and metastatic liver tumors including those from colon,[15] BH relies on rapid shock-induced heating of the tissue[14] rather than initiation of rarefaction-induced cavitation and, therefore, requires lower acoustic pressure levels *in situ*[11] and allows for the use of more compact transducers[16–18] and conventional clinical HIFU systems.[19–22] This feature of BH makes it a promising technology for applications in organs with limited acoustic window and/or requiring endoluminal (eg, transrectal) approach, such as local ablation of CRC. Similarly to thermal HIFU setup, BH transducer for CRC treatment can potentially be inserted transrectally — in a similar way as performed in a recently developed preclinical system for BH ablation of prostate tumors.[17,18,23,24] Such local US-based and US-guided ablation technique as BH could serve as a non-invasive non-thermal treatment modality for inoperable CRC[8] (for cytoreduction, obstruction treatment or initiating anti-tumor immune response[25,26]) and/or when chemotherapy is ineffective or is not recommended, as well as for local treatment of CRC metastases in other organs.

In our recent studies,[23,24,27–29] a sector BH transducer with the effective *F*-number (ie, ratio of the radius of curvature and aperture of the transducer)

$F\# = 0.83$ (focusing angle of 74.6°) working at a 1.5 MHz frequency has been shown to be capable of producing volumetric mechanical lesions in BH regimes in autopsy human tissues of various stiffness — brain tissues, breast cancer, uterine fibroids, and prostate tumors. However, a newer annular BH array has been recently designed and manufactured[30,31] to be capable of producing the acoustic field similar to that of a transrectal spoon-shaped transducer developed as part of a preclinical BH system for non-invasive treatment of prostate tumors in animal studies.[17,18] In order to be feasible transrectally, the array was designed to have a larger *F*-number of 0.9 (ie, smaller focusing angle of 67.5°) and higher US frequency of 2 MHz versus the one used in our previous human tissue studies, which typically leads to smaller BH lesions. The 2-MHz array has been recently shown to be feasible for mechanical liquefaction of large human hematomas in vitro, which represent the softest tissue model and, therefore, the most sensitive to BH. CRC tissue, however, is expected to be stiffer and, thus, more resistant to BH, and the possibility of BH fractionation of CRC has not been experimentally studied yet.

The goal of this study, therefore, was to experimentally demonstrate the feasibility of the BH approach for mechanical ablation of human colon cancer *ex vivo* under US guidance using the BH array imitating the field geometry of the preclinical transrectal transducer.

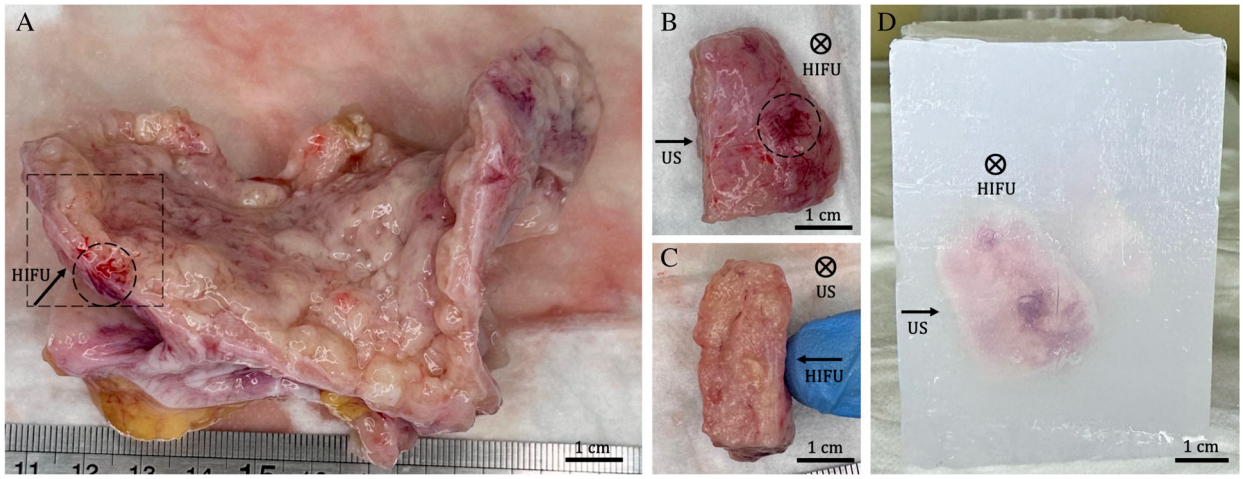
## Materials and Methods

### *Autopsy Tissue Preparation and Stiffness Measurements*

The tumor tissue (Figure 1A) was harvested following rapid autopsy 27 hours post-mortem of a de-identified 65-year-old male with a lifetime diagnosis of colon cancer (autopsy archive material, IRB exempt).

To confirm the clinical relevance of the autopsy material, its Young's modulus was measured through the serous membrane using shear wave elastography (SWE) on the Aplio i800 system (Canon, Japan) with a 14L5 sensor in a Thyroid preset in 3 circular areas of a 4-mm diameter with standard deviation calculated across the pixels within each circular area. Following SWE measurements, a tumor sample $\sim 23 \times 33 \times 16\ \text{mm}^3$ was dissected out of the autopsy material and subjected to 2.5 cycles of alternating degassing (30 minutes each) and compression (15 minutes each). The sample was then rapidly embedded into an acoustically transparent 1.5% agarose gel (UltraPure Agarose, Invitrogen, USA; density $\rho = 1019\ \text{kg/m}^3$, speed of sound $c = 1484$ m/s,

**Figure 1. A**–**C**, Surgically resected human sigmoid colon cancer before (**A**) and after (**B**,**C**) dissection of the sample for BH treatment. **D**, The cancer sample embedded into agarose gel. Dashed box in (**A**) roughly outlines the area dissected out for further BH (**B**,**C**). Dashed circle in (**A**) and (**B**) outlines the reference hemorrhaging to compare. HIFU is from the observer (⊗) in (**B**,**D**) and from the right in (**C**); ultrasound (US) imaging is from the observer in (**C**) and from the left in (**B**,**D**). BH, boiling histotripsy; HIFU, high-intensity-focused ultrasound.



absorption coefficient $\alpha = 0.001$ Np/cm at a 1 MHz frequency) at 43°C and attached to a 3-dimensional positioning system (UMS3, Precision Acoustics, UK) in a tank of degassed water at 34°C for further BH treatment (Figure 1, B–D).

### *Boiling Histotripsy Treatment*

BH exposure in the tumor sample was performed using a 2-MHz annular array (Figure 2A) described in detail in[30,31] three out of 12 outer ring elements of the array were disabled resulting in a 67.5° focusing angle ($F\# = 0.9$) to imitate the field geometry of the transrectal spoon-shaped transducer developed as part of a preclinical BH system for non-invasive treatment of prostate tumors (Figure 2, D–F).[17,18] Pre-treatment planning and in-treatment guidance were performed using B-mode US imaging (Figure 2A) with L7-4 probe (ATL, Philips, USA) driven by Verasonics V1 system (Kirkland, WA, USA).

Volumetric BH lesion in the tumor sample was produced by merging single lesions generated by sonication of a 3-dimensional grid of 5 × 5 × 2 foci with a 1-mm step (Figure 2B) as was successfully performed in recent human prostate and leiomyoma studies.[23,24,27] Note that the lesion was deliberately produced to fit within the tumor sample but not fractionate the tumor entirely in order to evaluate the BH-induced damage in comparison with the surrounding intact tumor structure. Hundred and fifty pulses of 1-ms duration each were delivered into each of the foci with a 0.1-second period, that is, 1% duty cycle. The electrical voltage (65 V) applied to the control system of array elements was chosen 30% above the threshold voltage (50 V), determined experimentally by gradually increasing the voltage

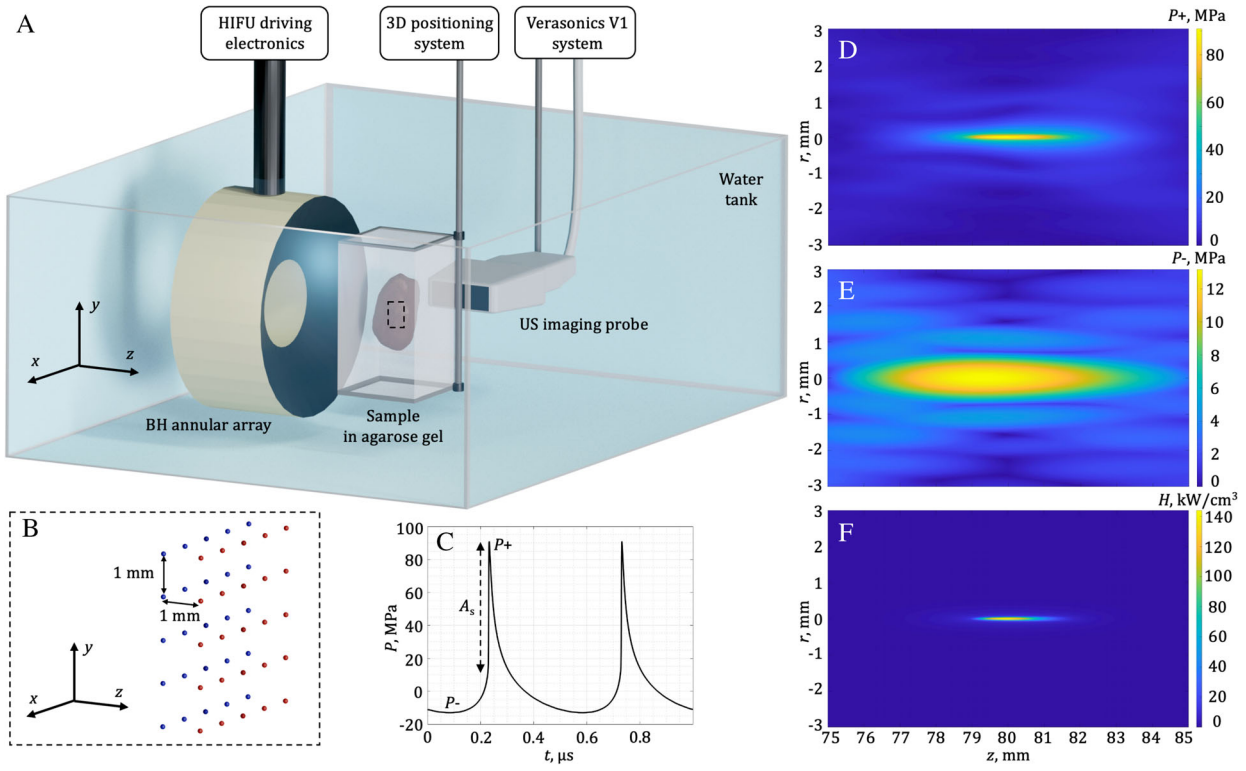



**Figure 2. A**, Experimental setup for *ex vivo* BH treatment of agarose-embedded human sigmoid colon cancer sample. Dashed box indicates ablated volume. **B**, Two layers of foci sonicated to merge single BH lesions into a volumetric lesion in the cancer sample. Red layer was sonicated first, then moving to the blue layer. **C**, Two cycles of numerically modeled pressure waveform at an average depth of focus in situ (8 mm). **D**–**F**, Numerically modeled 2-dimensional distributions of peak positive pressure $P+$ (**D**), peak negative pressure $P-$ (**E**) and density of heat sources $H$ (**F**) in the focal zone of the BH array. BH, boiling histotripsy.

until a hyperechoic spot appeared on US imaging indicating tissue boiling at the focus.

To characterize the US field generated in the tumor sample, nonlinear simulations were performed using the "HIFU beam" software[30–32] with boundary conditions based on acoustic holography measurements. The simulations were performed in a flat-layered medium "water – intestine" with the acoustic parameters of intestine taken from[33]: density $\rho = 1088$ kg/m$^3$, speed of sound $c = 1500$ m/s, nonlinearity coefficient $\beta = 4.03$, diffusivity coefficient $\delta = 4.33$ mm$^2$/s, absorption coefficient $\alpha = 0.115$ Np/cm at a 2 MHz frequency, with power law $\nu = 1.1$. Peak positive and negative acoustic pressures and shock amplitude at the selected voltage at the focus *in situ* estimated in numerical modeling were $P+/\ P-/\ A_s = 91/-13/78$ MPa, respectively (Figure 2C). The peak density of heat sources $H$ at the focus *in situ* was 144 kW/cm$^3$ (Figure 2F); the acoustic output power and initial intensity at the transducer surface within the pulse were $W_0 = 126$ W and $I_0 = 2.7$ W/cm$^2$, respectively.

### *Histological Examination of the BH Outcome*

Post-treatment, the resulting BH lesion was analyzed on US imaging, then the sample containing the lesion was marked with histological inks (HistoSafe, Russia) along and transverse to the imaging plane, fixed in 10% neutral buffered formalin (Labiko, Russia), processed for histological analysis and stained with hematoxylin–eosin (H&E) and Masson's trichrome (MT) stains. The resulting histological specimens were analyzed by certified pathologists (N.D. and P.M.) to determine the type of tumor and analyze the BH outcome. The treatment rate was estimated as the product of the 3 lesion dimensions measured either on US imaging or in histological images divided by the treatment time.

## Results

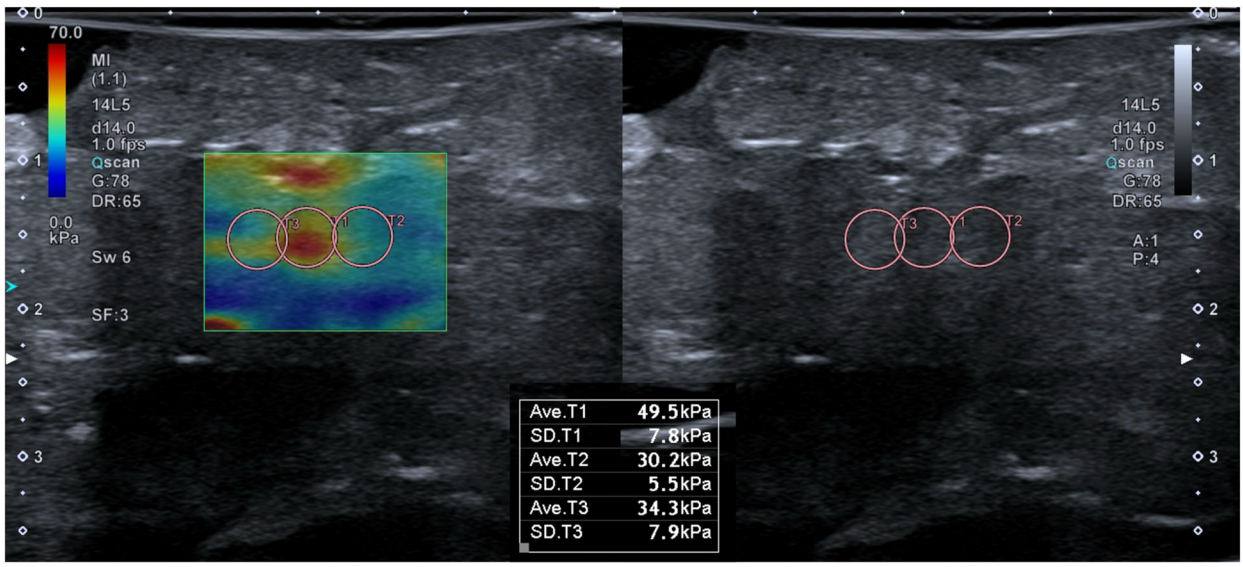
### *Stiffness of Human Colon Cancer*

SWE measurements confirmed the clinical relevance of the autopsy material (Figure 3): the Young's modulus values measured in the autopsy colon tumor ($38 \pm 10$ kPa) were higher than that typical for a healthy intestinal wall and were within the range clinically observed in colorectal adenocarcinoma.[34]

### *BH Treatment Process*

Pre-treatment targeting and real-time control of BH treatment were successfully performed using diagnostic US (Figure 4, A–C): the tissue appeared homogeneous pre-treatment (Figure 4A), then a hyperechoic

**Figure 3.** SWE image of the sigmoid colon cancer specimen acquired using Aplio i800 system with linear 14 L5 probe before dissection of the sample for further BH. BH, boiling histotripsy.



spot was observed in the treated area during the treatment (Figure 4B) and then it became hypoechoic immediately post-treatment (ie, right after the volumetric treatment was completed) indicating successful BH-induced liquefaction of the tumor tissue (Figure 4C). The hypoechoic lesion on US imaging corresponded well with the observed fractionated area after formalin fixation and bisection of the sample along the imaging plane (Figure 4D) as well as with the histologically confirmed fractionated zone (Figure 5). Volumetric treatment rate estimated from US imaging was 18.3 $mm^3$/min.

### *Histological Outcome*

Both H&E and MT staining of the tumor section containing the cross-section of the BH lesion (Figure 5) confirmed the diagnosis of mucinous colorectal carcinoma: large fields of mucin with tumor elements in the stroma of connective tissue and smooth muscle fibers were present outside the treated area (Figure 5, Ai and Bi). Within the treated area, histology confirmed BH-induced fractionation of tumor tissue: shapeless fractionated tumor cells were observed (Figure 5, Aii and Bii), as well as fragmentation of collagen fibers and homogenization of the tumor stroma (Figure 5, Aiii and Biii). A sharp boundary between the fractionated and intact tumor tissue was observed both along the HIFU axis (Figure 5, Av and Bv) and transverse to it (Figure 5, Aiv and Biv). The volumetric treatment rate estimated from histology was 12.5 $mm^3$/min—lower than estimated from US imaging due to the known shrinkage effect during formalin fixation.[35]

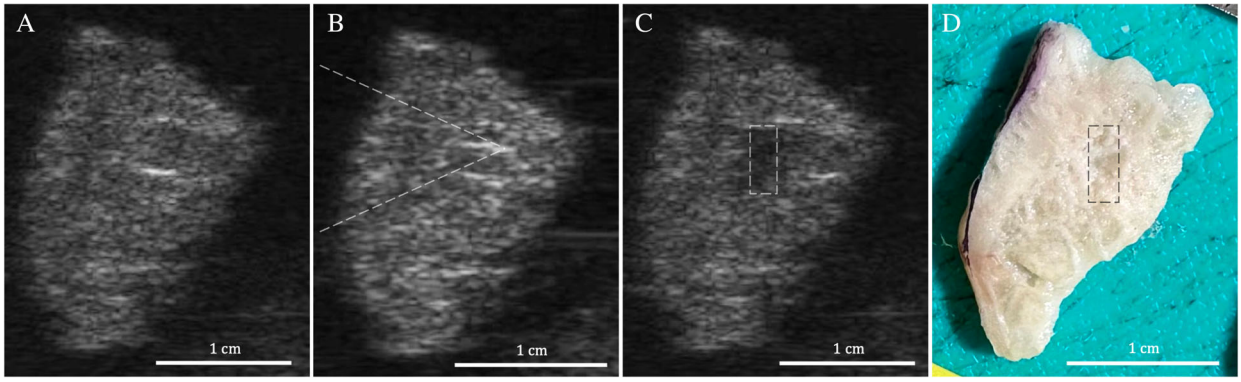
## Discussion

This study is the first to demonstrate the feasibility of BH to non-invasively mechanically (ie, non-thermally) fractionate human colon cancer *ex vivo* into sub-cellular debris under US-imaging guidance as an alternative to standard surgical resection and more recent non-invasive thermal HIFU ablation currently undergoing clinical trials.

BH treatment was successfully planned and guided in real time using conventional diagnostic US. Histology confirmed BH-induced homogenization of mucinous colorectal adenocarcinoma which is the second most common type of colon cancer after adenocarcinoma NOS (not otherwise specified). There is no prognostic difference between mucinous carcinoma and adenocarcinoma NOS but the former has a relatively poor response to systemic treatment. Therefore, it is important to develop a method for local destruction of mucinous carcinoma.

The treatment rate achieved in this pilot study was 18.3 $mm^3$/minute based on US imaging and 12.5 $mm^3$/minute based on histology (ie, lower due to the known shrinkage effect during formalin fixation[35]), which is higher than that obtained in recent *ex vivo* studies on human leiomyoma[27] and prostate tissues[23] due to their higher collagen content in the

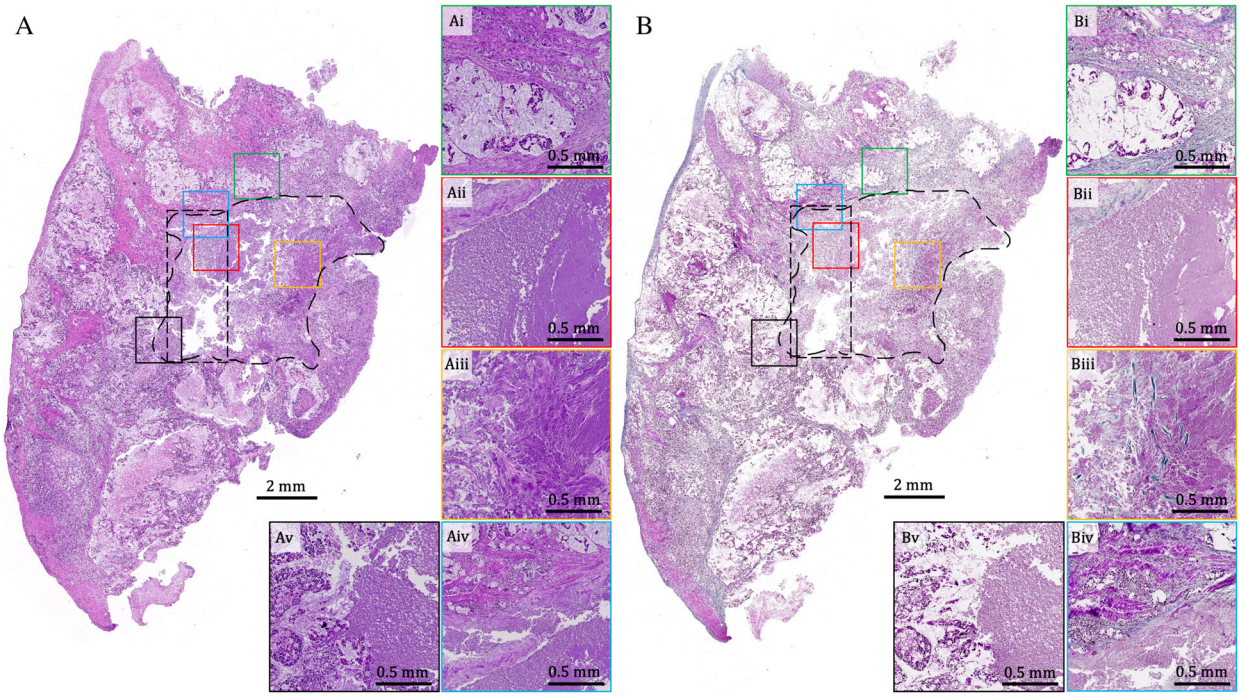
**Figure 4.** *Ex vivo* sigmoid colon cancer sample: B-mode ultrasound images (**A**) before, (**B**) during, and (**C**) 10 min after the BH treatment; (**D**) photo of the sample after formalin fixation and bisection along the imaging plane. HIFU incident from the left. Dashed lines in (**B**) indicate the BH beam geometry; dashed boxes in (**C**,**D**) indicate the target area (5 mm × 2 mm). BH, boiling histotripsy; HIFU, high-intensity-focused ultrasound.



stromal compartment as compared to that of colon cancer. The achieved treatment rate was lower than that clinically obtained in thermal HIFU ablation studies[7]; however, optimization of the BH protocol, that is, pulse duration, number of pulses per focus, step size between foci, etc., was beyond the scope of this pilot study and can potentially increase the treatment rate for BH ablation of CRC tumors. The treatment parameters here were deliberately chosen to be the same as those previously used for BH in uterine and prostate tumors,[23,27] despite their higher stiffness versus CRC, to ensure successful disruption of CRC in this pilot study. Our findings indicate that, since the disrupted tissue volume significantly exceeded the target zone, the treatment parameters were excessive and can be optimized to accelerate the treatment and achieve clinically relevant ablation rates through reducing the number of pulses per focus, increasing the pulse duration to produce larger lesions, and increasing the step size between foci. Moreover, such complete ablation may not be necessary in clinical settings, where disruption of cancerous cells only, which are more susceptible to histotripsy versus fibrous stroma of the tumor, can be sufficient.[36] Partial volumetric ablation forming non-perfused tissue volumes may be another way of accelerating the treatment while still creating a non-viable environment for cancer cells.[37]

The BH annular array was used here in a configuration that imitates the existing transrectal BH transducer with an incorporated diagnostic US probe developed within the preclinical BH system for prostate tumor ablation.[17,18,31] Successful BH treatment of human colon tumor *ex vivo* with this array here implies that BH ablation of human CRC can potentially be performed endoscopically through the rectum in clinical settings similarly to thermal HIFU treatments in.[7,8] Such a non-invasive local ablation technique could be useful in the cases of inoperable CRC[8] (for cytoreduction, obstruction treatment and initiation of anti-tumor immune

**Figure 5.** Histological specimens of the human sigmoid colon cancer after volumetric BH treatment *ex vivo*, stained with H&E (**A**–**Av**) and MT (**B**–**Bv**). **A**,**B**, Histotopograms of the treated sigmoid colon cancer specimen, (i) intact tumor tissue adjacent to the treated area; (ii) liquefied content of the BH lesion, (iii) injured tumor stroma in the distal part of the lesion; (iv) side border of the lesion; (v) frontal border of the lesion. HIFU incident from the left. Dashed boxes in (**A**–**Ai**, **B**–**Bi**) represent the target area (5 mm × 2 mm). BH, boiling histotripsy; H&E, hematoxylin and eosin; MT, Masson's trichrome; HIFU, high-intensity-focused ultrasound.



response[25,26]) and/or when chemotherapy is ineffective or is not recommended, as well as to locally treat CRC metastases in other organs. In the case of primary CRC treatment, the transrectal approach would minimize the thickness of intervening tissues between the transducer and target tissue as opposed to the transabdominal approach, thus minimizing any aberration and attenuation of the US beam, yet preserving the non-invasiveness of the procedure. Besides the direct ablation effect, it has been reported that histotripsy-induced tumor fractionation can enhance anti-tumor immune response by releasing cancer-associated antigens and activating the cancer immunity cycle.[25,26] This can be particularly useful in the treatment of primary CRC tumors that are inoperable due to the risk of bleeding or perforation leading to peritonitis: partial instead of whole-tumor fractionation with BH can potentially be performed to initiate immune response and promote abscopal effect on the residual part of the primary tumor and on distant untreated metastases.[25,26] However, evaluation of the BH feasibility for immunomodulation in CRC management requires further extensive investigations *in vivo*. Moreover, since histopathology of primary and metastatic CRC tumors are expected to be similar,[38] our results imply that BH can also be feasible for non-invasive non-ionizing US-guided fractionation of CRC metastases in other organs.

## Conclusion

The successful pilot experiment described here is the first to demonstrate the feasibility of the BH approach for US-guided non-invasive mechanical (ie, non-thermal) fractionation of human CRC *ex vivo* (sigmoid colon mucinous adenocarcinoma). The obtained results indicate the potential of BH for non-invasive US-guided treatment of inoperable CRC and its metastases and encourage further research aimed at data collection and protocol optimization for the further implementation of BH into clinical practice.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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