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Original Contribution

Treatment of Liver Metastases With Focused Ultrasound and Microbubbles in Patients With Colorectal Cancer Receiving Chemotherapy

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Objective: Pre-clinical trials have obtained promising results that focused ultrasound (FUS) combined with microbubbles (MBs) increases tumor uptake and the therapeutic effect of drugs. The aims of the study described here were to investigate whether FUS and MBs could improve the effect of chemotherapy in patients with liver metastases from colorectal cancer and to investigate the safety and feasibility of using FUS + MBs.

Methods: We included 17 patients with liver metastases from colorectal cancer, selected two lesions in each patient's liver and randomized the lesions for, respectively, treatment with FUS + MBs or control. After chemotherapy (FOLFIRI or FOLFOXIRI), the lesions were treated with FUS (frequency = 1.67 MHz, mechanical index = 0.5, pulse repetition frequency = 0.33 Hz, 33 oscillations, duty cycle = 0.2%–0.4% and MBs (SonoVue) for 35 min). Nine boluses of MBs were injected intravenously at 3.5 min intervals. Patients were scheduled for four cycles of treatment. Changes in the size of metastases were determined from computed tomography images.

Results: Treatment with FUS + MBs is safe at the settings used. There was considerable variation in treatment response between lesions and mixed response between lesions receiving only chemotherapy. There is a tendency toward larger-volume reduction in lesions treated with FUS + MBs compared with control lesions, but a mixed response to chemotherapy and lesion heterogeneity make it difficult to interpret the results.

Conclusion: The combination of FUS and MBs is a safe, feasible and available strategy for improving the effect of chemotherapy in cancer patients. Therapeutic effect was not demonstrated in this trial. Multicenter trials with standardized protocols should be performed.

Introduction

Colorectal cancer is the second most common cause of cancer death worldwide [1]. Because of the portal circulation, the liver is the most common site for metastases, and 25% of these patients develop liver metastases during their disease course [2]. Chemotherapy is used in both curative and life-prolonging setting for patients with liver metastases. For a sustained anti-tumor effect, all malignant cells need to be exposed to a sufficient dose of the anti-neoplastic agent. In fact, a disappointingly small fraction of systemically administered drugs reach their desired target. The limited number of drug distribution studies performed in humans reveal very low uptake in solid tumors [3,4].

Treatment of solid tumors with cytotoxic agents is greatly restricted by their abnormal tumor microenvironment [5–7]. Physical factors in the tumor microenvironment promote treatment resistance at four recognized levels: the vascular system, the vessel wall, the extracellular matrix (ECM) and the tumor cell membrane. Drugs, along with oxygen and nutrients, need to be supplied via a functional vascular system. Tumor vessels are abnormal in structure and function—large intra- and intercellular openings, pathologic basement membrane and reduced pericyte coverage lead to hyper-permeability [5]. The transport of drugs across the capillary wall and through the ECM is governed by diffusion caused by the concentration gradient and convection caused by the pressure gradient. The capillary hyper-permeability in combination with the lack of functional lymphatics, called the enhanced permeability and retention effect, results in a high interstitial fluid pressure, which limits extravasation of drugs across the capillary wall and penetration through the ECM. Because of the high interstitial pressure, diffusion becomes the major transport mechanism. The ECM, consisting of a network of fibrillar collagen embedded in a hydrophilic gel of glycosaminoglycans

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(GAGs), is reported to limit diffusion of drugs. The collagen content is correlated to interstitial diffusion of macromolecules, whereas GAG content does not exhibit such a clear correlation [6,8].

Various strategies have been explored to increase the uptake and effect of antineoplastic drugs. For FUS combined with MBs, promising results for improving local treatment effect in both pre-clinical [7,9–13] and clinical [14–16] trials have been reported. Our own research group recently published pre-clinical results revealing improved uptake of conventional chemotherapy into orthotopic pancreatic tumors in mice [12]. For colorectal cancer in particular, Ingram et al. [17] and Lin et al. [18] reported increased therapeutic effectiveness in subcutaneous colorectal mouse models.

The aim of our trial was to investigate the effect of chemotherapy in combination with FUS and MBs on selected liver metastases in patients with colorectal cancer. The trial is designed in accordance with today's clinical practice and national guidelines. Commercially available, well-established drugs and MBs, as well as clinically available ultrasound transducers, were used.

The primary endpoint was volume change of pre-selected FUS-treated liver metastases versus control metastases receiving only chemotherapy. A secondary aim was to evaluate safety of this experimental procedure and gain experience on practical feasibility.

Methods

Patient recruitment and collection of clinical data

The trial was approved by the regional ethics committee (REK) under REK 2018/30 and by the Norwegian Medicines Agency (SLV) under EudraCT No. 2018-002814-11. All procedures were conducted according to international Good Clinical Practice (GPC). Seventeen volunteer patients were recruited from the outpatient Cancer Clinic at St. Olav's Hospital–Trondheim University Hospital over a 39-mo period from February 2019 to May 2022. All patients signed an informed consent. Inclusion criteria were as follows: histologically verified colorectal carcinoma, two or more liver metastases, eligibility for first-line treatment with irinotecan-based chemotherapy regimens combined with epidermal growth factor receptor (EGFR) inhibitor when indicated, age ≥18 y and <90 y. Exclusion criteria were known contraindications to SonoVue (Bracco International BV, Amsterdam, Netherlands) and a hematological bleeding status comprising a hemoglobin (Hb) < 8 g/dL, thrombocytes (TC) $<80 \times 10^9$/L, activated partial thromboplastin time (APTT) >45 s, International Normalized Ratio (INR) >1.5; patients considered for surgical excision of liver metastases and pregnant patients are also excluded. Clinical data were collected from patient records. Baseline characteristics of patients are given in Table 1.

Table 1
Baseline characteristics at inclusion (n = 17)

Variable	Mean (min–max) [normal value]
Age (y)	66.5 (45–80)
Sex (male/female)	12/5
Body mass index	25.6 (19–32)
Primary tumor location	
Colon/rectum	14/3
Right/left[a]	8/9
Intact primary tumor (yes/no)	10/7
Diameter of metastases treated with FUS + MBs at baseline (mm) Diameter of control metastases at baseline (mm)	30 (13–47) 30 (11–68)
C-Reactive protein (mg/L)	60 (<5–140) [<5 mg/L]
Lactate dehydrogenase (U/L)	616 (178–2146) [106–333 U/L]
Carcinoembryonic antigen (μg/L)	646 (3–7425) [<2.5 μg/L]
Carbohydrate antigen 19-9 (kIU/L)	2519 (2–20,258) [<37 kIU/L]
Albumin (g/L)	41 (35–46)
BRAF+	3/17
KRAS+	10/17
NRAS+	2/17
Microsatellite instability	1/17

BRAF, v-raf murine sarcoma viral oncogene homolog B1; FUS–MBs, focused ultrasound combined with microbubbles; KRAS, Kirsten rat sarcoma viral oncogene homolog; NRAS, neuroblastoma ras viral oncogene homologue.
[a] Right/left border at splenic flexure.

Internal randomization of metastases

Two liver metastases were selected on computed tomography (CT) in different segments of the liver and named '1' or '2'. Criteria for selection of the two lesions were easy to demarcate and recognize on subsequent CT image and far apart from each other to avoid ultrasound exposure of the control lesion. The selected lesions were randomized 1:1 by software in a web-based Clinical Report Form (WebCRF3, NTNU, Norway) and allocated to the arms "experimental" and "control." Selection of lesions for randomization is illustrated in Figure 1b.

Chemotherapeutic and MB dosage

Patients received intravenous standard chemotherapy in the outpatient clinic according to hospital practice and Norwegian national guidelines [19]. Sixteen patients received the regimen FOLFIRI and each individual drug was administered sequentially in the order irinotecan 180 mg/m^2 (Accord Healthcare, Middlesex, UK) and calcium folinate 400 mg/m^2 (Pfizer, Zaventem, Belgium) (both with an infusion time 1 h); fluorouracil 400 mg/m^2 (Accord Healthcare, Middlesex, UK) as a bolus infusion <5 min; and fluorouracil 2400 mg/m^2 infused over 46 h. FUS and MBs were administered as soon as practically feasible after the fluorouracil bolus. After completion of ultrasound treatment, a 46 h infusion of fluorouracil started.

One patient received the regimen FOLFOXIRI, consisting of calcium folinate 200 mg/m^2, oxaliplatin 85 mg/m^2 (Fresenius Kabi, Friedberg, Germany) and irinotecan 165 mg/m^2. The infusion time was 1 h for all drugs. Fluorouracil 3200 mg/m^2 was given as a 46 h infusion. Experimental treatment was administered as soon as possible after irinotecan and before the 46 h fluorouracil infusion.

Two patients with wild-type KRAS (Kirsten rat sarcoma viral oncogene) tumors with indication for EGFR inhibitor, received panitumumab 6 mg/kg (Amgen Technology, Dublin, Ireland) infused over 1 h, followed by FOLFIRI.

The MB contrast agent SonoVue was administered as 1 mL intravenous boluses every 3.5 min after the start of ultrasound treatment and repeated nine times. All MB boluses were followed by a 10 mL NaCl intravenous bolus.

Ultrasound configuration/experiment configuration

The ultrasound scanner Vivid E9 (VE96975, GE Vingmed Ultrasound AS, Norway) was used both for imaging and for therapeutic ultrasound with software adjustments for treatment setting. A 2- to 5-MHz abdominal curvilinear probe (4C-D, GE Healthcare, GE Parallel Design Inc., 215041WX5, China) was used for imaging and identification of lesions, and a 1.5- to 4-MHz 2-D matrix probe (4V-D, GE Healthcare, GE Parallel Design Inc., 144582PD4, USA) was used for treatment. An electrocardiogram (ECG) trigger generator (Model 7800, Ivy Biomedical, Inc., Branford, CT, USA) was connected to ensure uniform and reproducible sonication over the target lesion. An optical tracking device (Polaris Spectra Position Sensor, ID 11800, NDIgital, Waterloo, ON, Canada) was used to determine, in real time, the 3-D location of each probe. Therapy setup is illustrated in Figure S1 (online only).

Navigation procedure

The included patients had numerous metastatic liver lesions. To ascertain that the correct lesion selected on the CT image for FUS was exposed to ultrasound and that the control lesion was not sonicated, a

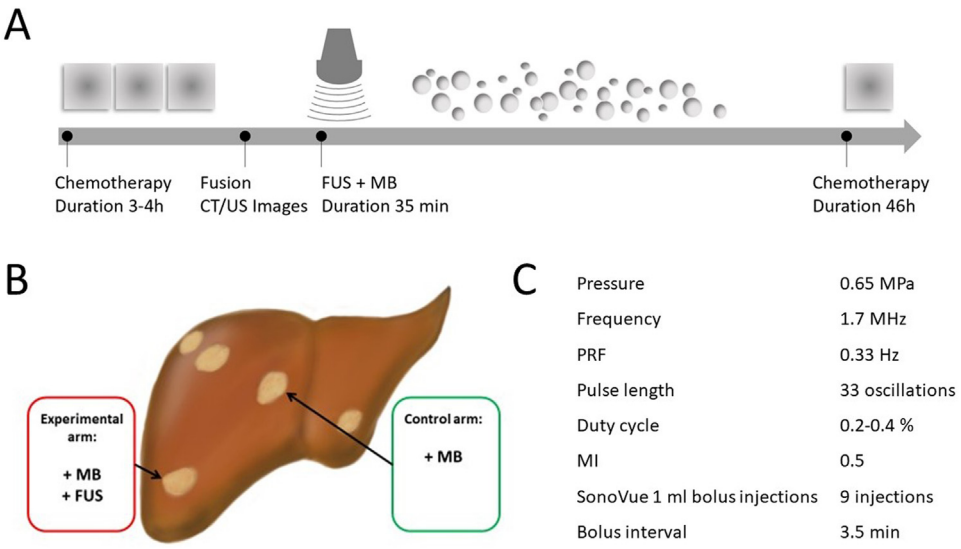

Figure 1. Experimental treatment, study design and sonication parameters. (a) Timeline of experimental treatment. Combination chemotherapy (FOLFIRI/FOLFOXIRI) is administered before fusion of CT/US images and start of FUS + MBs. One-milliliter boluses of MBs were injected intravenously every 3.5 min followed by FUS exposure, which lasted 35 min. The 46 h infusion of fluorouracil began after completion of the experimental treatment. (b) Internal randomization. The lesion randomized for experimental treatment (*red box*) is treated with FUS in addition to systemic chemotherapy and MBs. The control lesion (*green box*) is treated with systemic chemotherapy and MBs only. (c) Ultrasound parameters: pressure, frequency, PRF, pulse length, duty cycle, MI and SonoVue bolus injections. CT, computed tomography; FUS, focused ultrasound; MBs, microbubbles; MI, mechanical index; PRF, pulse repetition frequency; US, ultrasound.

custom-made navigation procedure overlying the CT and ultrasound image was developed. We used a plug-in module in the CustusX platform [20], a system developed for image-guided interventions. The navigation procedure is described in the Supplementary Material (online only).

After CT and ultrasound image fusion, the 4V probe was used to perform the treatment and was fixed in position with an adjustable arm with the lesion randomized for ultrasound treatment in the center of the field of view (FOV). To correct for patient movement during treatment, the probe position was monitored. The CustusX platform and optical tracking ensured proper positioning (Fig. S2, online only).

Sonication of tumors

The 4V-D probe used for ultrasound treatment has a 2-D matrix array that enables focusing and scanning both in the azimuth direction, which is the scan plane for a conventional 1D array, and in the elevation direction, which is the plane transverse to this conventional scan plane. Thus, we were able to sonicate a full 3-D tumor volume. Ultrasound gel was applied as a coupling medium between the transducer and the patient's skin. The 4V-D therapy probe was fixed in position with an adjustable arm.

To select and sonicate a 3-D volume, the 3-D color flow application on the scanner was used. A region of interest (ROI), which is selectable on the screen of the scanner, was placed around the tumor to be treated. The transmit and receive configuration of the scanner within the ROI is partly determined on the basis of the location and shape of the indicated ROI, and the selected 3-D volume is split into several slices in the elevation direction. The multibeat mode of the 3-D color flow application allows the user to manually set several parameters (*e.g.,* pulse repetition frequency, number of elevation slices, ECG trigger frequency). This mode was used to obtain equal ultrasound exposure for each tumor regardless of tumor size and depth, where freely selectable parameters were individually set for each patient. To enable equal ultrasound exposure with the indicated probe, we could only include tumors occurring closer than 65 mm (center of tumor) in depth and with diameters less than 45 mm. The duty cycle of the ultrasound probe was 0.2%–0.4 % depending on the tumor size. The tumors treated with FUS and MBs were all located superficially within the liver, meaning that there was little normal liver parenchyma between the ultrasound probe and the tumor. The pulse length for each therapy pulse was set to 33 oscillations, which was the maximum allowed by the system, and the transmit frequency was 1.67 MHz. For a given elevation slice, we used numerous transmit beams to scan the elevation slice repeatably in the azimuth direction with a high pulse repetition frequency (*e.g.,* 3 kHz). The mechanical index (MI) displayed by the scanner was 0.5 for all tumors, and the applied peak negative pressure for all tumors was 0.65 MPa. It is expected that the MBs within a sonicated elevation slice will burst at the indicated MI. To allow for reperfusion of MBs within a given subvolume, we ensured a fixed time of 3 s, resulting in a pulse repetition frequency of 0.33 Hz, between each sequence of sonication of a given tumor elevation slice. The same operator located the tumor and positioned and controlled the probe for all treatments.

Focused ultrasound and MBs were administered, on average, 45 ± 14 min (mean ± standard deviation [SD]) after completed infusion of FOLFIRI or FOLFOXIRI on day 1. Included patients received in average 3.5 cycles of FUS + MBs.

Figure 1a is the timeline for experimental treatment. Acoustic parameters are provided in Figure 1c.

Response evaluation and tumor volume measurements

Computed tomography was performed with an 80 s fixed time delay after intravenous injection of the contrast agent Omnipaque or Visipaque (both from GE Healthcare AS, Nydalen, Norway), with a slice thickness of 3 mm. CT images were obtained twice for all patients: the first image within 3 wk before treatment start (baseline evaluation) and the second after the last treatment (response evaluation). Two patients had their baseline CT taken more than 3 wk before treatment start. Response evaluation was performed according to standard response evaluation criteria for solid tumors (RECIST 1.1 [21]) by radiologists at St. Olav's Hospital. Complete response (CR) indicates disappearance of all lesions, partial response (PR) indicates ≥30% decrease in sum of the longest diameter (SLD). Progressive disease (PD) indicates ≥20% increase in SLD compared with the smallest SLD in the study. Stable disease (SD) indicates neither PR nor PD. Percentage change of lesions given by the change in the longest diameter was calculated as the difference in the longest diameters (mm) between the post- and pre-treatment CT scans, divided by the diameter of the pre-treatment scan. Tumor volumes of

pre-selected lesions randomized for ultrasound treatment or control were measured using software by ITK Snap (Version 3.8.0). The percentage volume change was calculated as the volume (mm^3) difference between the post- and pre-treatment CT-scans divided by the volume of the pre-treatment scan. To examine the occurrence of mixed response to chemotherapy, volumes of three additional metastases in the liver not exposed to ultrasound were measured.

Recording of adverse events

Adverse events (AEs) were recorded based on Common Toxicity Criteria Version 5.0 (2017) and recorded from patient records.

Statistics

Graphs and statistical calculations were done by Graphpad Prism 8.0.1 (244) (San Diego, CA, USA). For statistical significance testing, two-tailed, unpaired *t*-tests with a significance level $p < 0.05$ and 95% confidence interval were used. The Kolmogorov–Smirnov test was used to test for a normal distribution.

Results

Patient baseline characteristics

We included patients with a mean age of 66.5 y. Seventy percent of included patients were males. The primary tumor was located in the colon in 82% of patients, and the majority of patients had intact tumors at the time of inclusion. Mean diameter (mm) of selected metastases before randomization was approximately equal for both ultrasound-treated and control groups, but varied between patients. Baseline characteristics are outlined in Table 1. The resulting group of included patients reflects the patient group well with respect to age and sex. For healthy individuals in Norway aged 65–74, 65% of males and 52% of females have a body mass index (BMI) in the range 25–30 [22]. The biomarker features of our patients are not reflective of a larger population according to published reviewed literature [23] for stage IV colorectal cancer. However KRAS+ is the most common mutation in our study group, as it is in the literature. The incidence of NRAS+ is 3%–5 % in the reviewed literature and 12% in the present study. The incidence of BRAF+ is 8%–12 % in the reviewed literature and 18% in the present study. Generally, the baseline characteristics are characterized by large variations.

Treatment regimens, delays and dose reductions

For different reasons delays in scheduled chemotherapy occurred, and dose reduction was necessary for some patients. These changes are indicated in Table 2. FUS and MBs were administered as a supplementary treatment after 93.8% of chemotherapy cycles.

RECIST evaluation

The time interval between baseline CT and CT for response evaluation was on average 68.8 ± 16 d. According to RECIST 1.1 criteria at response evaluation, 4 (23.5 %) patients achieved a PR, 2 (11.7 %) a PD and 11 (64.7 %) an SD. No patients achieved CR.

Effect of ultrasound and microbubbles

This study was designed to have volume change of metastasis as the primary endpoint. In addition, the percentage change in the longest diameter was calculated. Figure 2a illustrates the volume percentage changes in ultrasound-treated lesions versus pre-selected controls. There is a trend toward improved effect for ultrasound-treated lesions, but there is large variation, and the difference is not statistically significant.

Table 2
Treatment regimens

Treatment parameter	No. of treatments	No. of patients
Total number of chemotherapy cycles	64	17
Regimen		
FOLFIRI	53	14
Pan-FOLFIRI	7	2
FOLFOXIRI	4	1
Deferred treatment[a]		
0 d	32	
≤5 d	6	
>5 but ≤10 d	8	
>10 d	1	
Dose reductions		
35%	1	
30%	5	
25%	4	
20%	14	
10%	3	
0	37	
Total No. of focused ultrasound cycles[b]	60	17
Average time between chemotherapy and start of focused ultrasound (min)	45 (32–61)	
No. of focused ultrasound treatments per patient		
4		10
3		6
2		1

[a] Deferred treatment = number of days of delay of cycles 2–4 according to 14-day cycle schedule. Two delays were intentional. Total possible number of delays = 47.
[b] One treatment was not successful, owing to equipment failure.

The percentage change in longest diameter *ad modum* RECIST 1.1 is illustrated in Figure 2b. To compare the volume change in ultrasound-treated versus control lesions in the same patients, each patient is color-coded in Figure 3.

Responders in favor of FUS

Patients in which the treated lesions decreased more or increased less than those in selected controls were classified as responders. Twelve of 17 patients responded in favor of FUS by this definition. Results according to this definition are outlined in Table 3.

There was no significant difference between the two groups with respect to age, primary tumor location, operational status or BMI at baseline. The total number of days treatment was delayed and the average time gap between completed intravenous infusion and start of FUS + MBs were without significant difference. Reduction of dose of the therapeutic regimen was slightly higher in the responder group (13% vs. 6 %) and not statistically significant.

Simple linear regression of percentage volume response versus biochemical expression of tumor load (CEA, CA 19-9, CRP, LD) or nutritional status (BMI, albumin) revealed no correlation. There was no correlation between FUS responder status and any baseline characteristics.

Volumes of additional control lesions: mixed response to chemotherapy

In addition to the two pre-selected lesions (ultrasound-treated and control lesions), we selected three additional lesions and calculated their volume change after chemotherapy. Figure 3 illustrates the percentage volume changes for all lesions plotted for the different groups of selected lesions. The five lesions per patient have the same color or shape. Similarly, as for the pre-selected randomized lesions, the additional lesions (extra one to three) also exhibited a great variation in response. There was a tendency for the FUS-treated lesions to have a larger reduction in volume than the additional three lesions, as also seen for the pre-selected lesion (Fig. 2a). To see how the volumes of the five lesions changed for each patient, the volume change was plotted for each of the patients

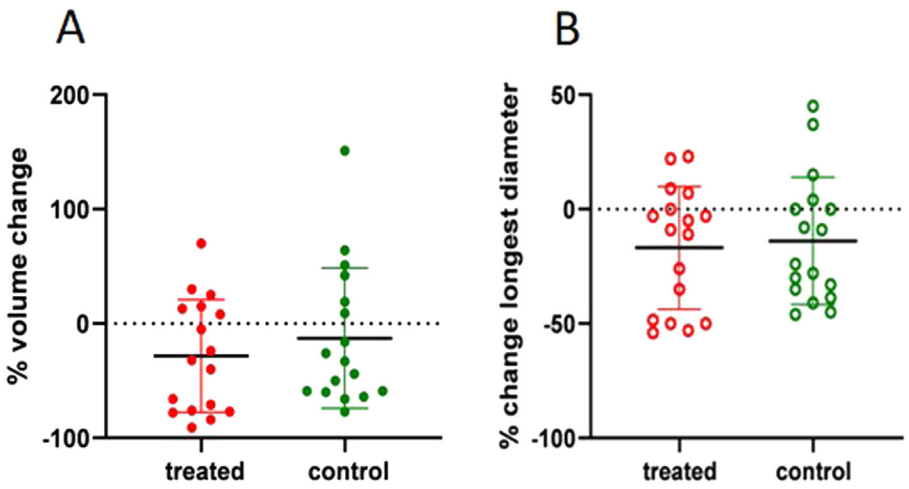

Figure 2. Percentage change in lesion volumes and longest lesion diameter. (a) Volume (mm^3) percentage change of treated (*red dots*) and control (*green dots*) lesions. (b) Longest diameter (mm) percentage change of treated (*red circles*) and control (*green circles*) lesions. Each circle represents one lesion. The *black line* represents the mean change. *Colored lines* are standard deviations.

numbered 1-17 (Fig. 4). There is a clear mixed response to chemotherapy; that is, lesions increased or decreased within the same patient.

Figure 4 illustrates that four patients had no or a very poor response (patients 3, 5, 7 and 15). Among these, patients 3, 5 and 15 are also mixed responders. Seven patients are clear mixed responders (1, 4, 5, 8, 9, 11 and 14). Six patients (2, 10, 12, 13, 16 and 17) are good responders. There is considerable variability also among good responders. Variability is greatest among the non-responders.

Safety: adverse events

All recorded adverse events (AEs) were indistinguishable from AEs caused by chemotherapy alone, such as bone marrow toxicity, nausea, diarrhea and cachexia. No unexpected AEs or serious AEs related to the ultrasound treatment were recorded. The skin in the contact area of the transducer during treatment was inspected immediately after each treatment. No petechial bleeding was observed. One patient stopped

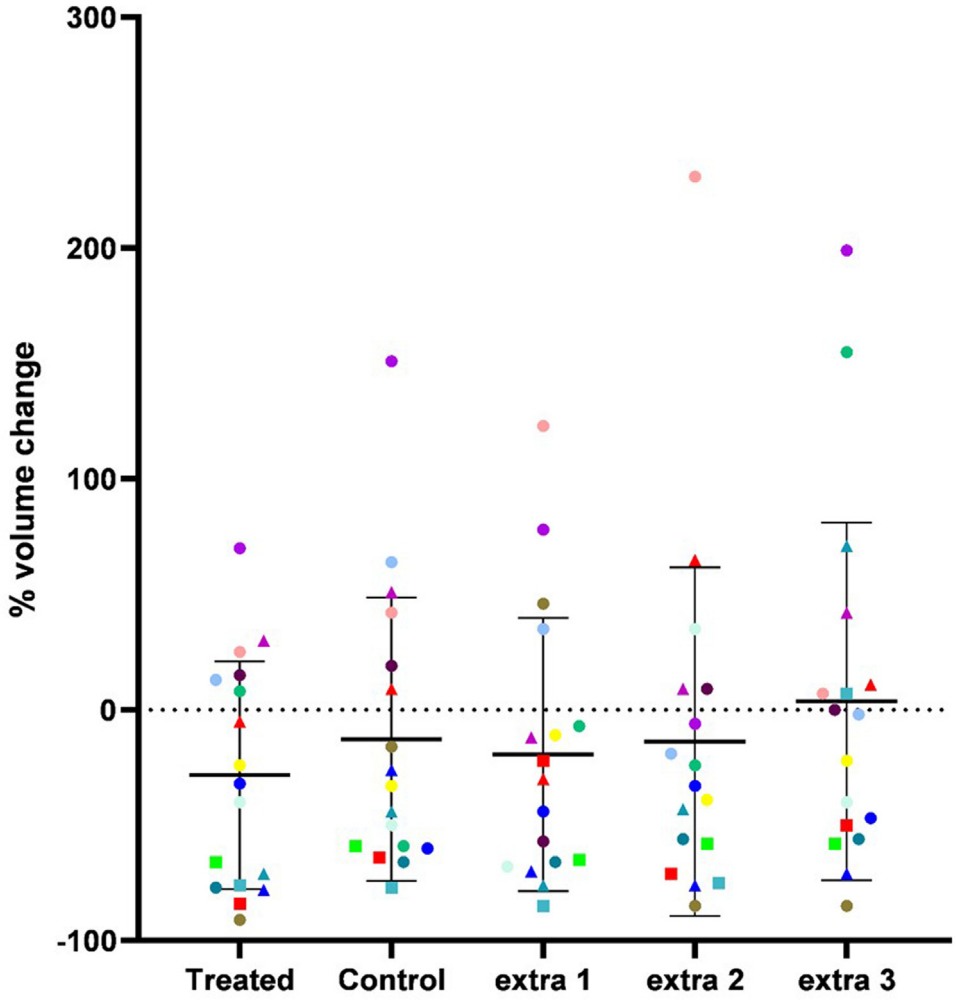

Figure 3. Volume percentage change for different groups of lesions. Ultrasound treated, pre-selected control and extra controls 1–3. Each color or shape represents individual patients. *Black lines* represent standard deviations. *Bold black lines* represent mean changes.

Table 3
Status classified according to defined responders or non-responders to FUS

Parameter	Responder to FUS	Non-responder to FUS
Number	12 (71%)	5 (29%)
Sex (% female)	25%	40%
Age (y)	67.3 ± 6.7	65.8 ± 6.3
Primary location (left)	50%	60%
Intact primary tumor (yes)	75%	80%
Body mass index baseline	25.8 ± 4.1	25.4 ± 3.5
Interval between chemo bolus and start of FUS (min)	46 ± 7.5	44 ± 8.9
Deferred treatments (d)	1.7 ± 1.1	1.6 ± 2.1
Dose reduction/cycle	13%	6%
Hospitalization (d)	4.8 ± 8.3	3.0 ± 5.1

FUS, focused ultrasound.

chemotherapy because of portal vein thrombosis, and one patient stopped treatment because liver steatosis developed. One patient, with an intact primary tumor, had a colon perforation after the fourth treatment. Average days of hospitalization for all patients, from the day of first treatment to 20 d after the last treatment, was 4.2 ± 7.5 d. Mean duration of hospitalization was 4.8 ± 8.2 d in the FUS responders versus 3.0 ± 5.1 d in the non-responders to FUS.

Duration of hospitalization during ongoing ultrasound treatment and until 20 d after treatment was as expected for this patient group. Our data suggest that treatment with FUS and MBs with the given ultrasound exposure and doses of MBs is safe.

By 1 March 2023, 49 mo after inclusion of the first patient, survival was 41% or 9.6 ± 6.4 mo.

Discussion

This clinical trial is one of very few investigating the effect of FUS + MBs in combination with chemotherapy in cancer patients. The results imply that treatment with FUS + MBs with the given ultrasound parameters and treatment regimen is safe and feasible for this group of patients. The trial did not reveal a significant effect of FUS + MBs with respect to tumor volume at evaluation after four cycles (2 mo) of treatment, although we could see a tendency toward increased effect on ultrasound-treated metastases.

Earlier clinical trials using FUS and MB to enhance treatment effect have focused mainly on safety and feasibility and have, in agreement with our results, also determined that the procedure is safe. Dimcevski et al. [14] reported that FUS and MBs contributed to improved performance status in 10 treated patients with locally advanced pancreatic adenocarcinoma. The improved performance status resulted in a significantly higher number of chemotherapy cycles received, compared with historical controls. Survival was also significantly improved. Zhou et al. [16] reported reduced size of primary breast cancers after neoadjuvant treatment combined with FUS + MBs in 10 treated patients, compared with controls. Rix et al. [24] treated 6 patients receiving neoadjuvant chemotherapy for breast cancer with FUS + MBs, resulting in no significant change in vascularization evaluated by tumor perfusion and delayed tumor size reduction, compared with controls.

The reasons for not observing any effect of FUS + MBs in our study might be related to the ultrasound parameters applied, the timing between the chemotherapy and ultrasound treatment and the mixed response to chemotherapy. These factors are discussed below.

All three clinical studies mentioned above used SonoVue and the injections were repeated six to nine times. Both Dimcevski et al. [14] and Zhou

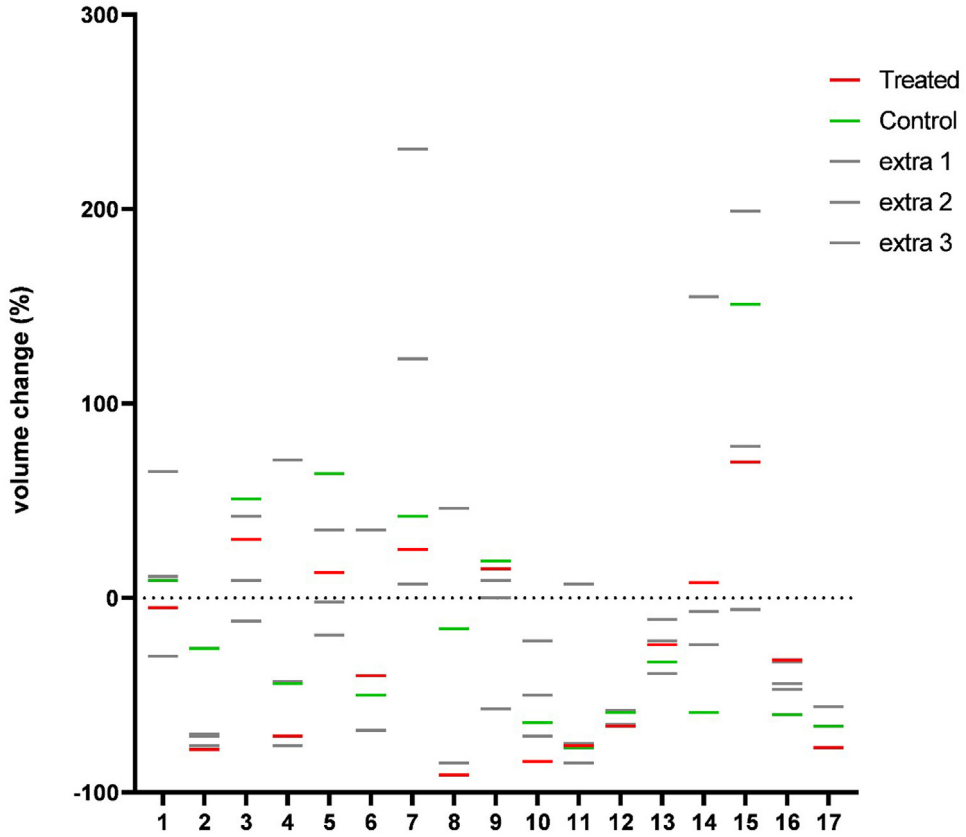

Figure 4. Volume percentage change of five lesions for all individual patients 1–17. Each colored line represents one lesion. *Red lines* represent pre-selected, randomized ultrasound-treated lesion. *Green lines* represent pre-selected, randomized control lesion. *Gray lines* represent extra lesions.

et al. [16] applied an ultrasound frequency close to the resonance frequency (1.9–4.0 MHz), consistent with our study. Their MI (0.2) was somewhat lower than what we used, assuming the MI reported is the MI displayed by the ultrasound system. The duty cycle applied by Dimcevski et al. [14] was similar to ours, 0.3%. Zhou et al. [16] did not report the duty cycle. Rix et al. [24] had a different approach applying a higher frequency of 7 MHz and an MI of 0.8, in parallel with chemotherapy. They studied potential ultrasound-induced changes in perfusion, as ultrasound and microbubbles at high MI have been reported to induce vascular shutdown and improve therapeutic outcome in tumors growing in mice [25].

It is not clear which MI is optimal to improve drug delivery and therapeutic outcome. A consequence of a high MI is more disruption of MBs, as reported by Snipstad et al. [26]. This elicits a need for time to allow for reperfusion of MBs within the target volume. A low MI will permit prolonged oscillations of MBs and more time to achieve a biological effect. On the other hand, a higher MI will cause a more vigorous effect from the MBs, which might be needed to achieve the biological effect we desire.

The ultrasound parameters used in our study are partly based on experience from pre-clinical studies and partly limited by the diagnostic ultrasound system used. The MI of 0.5 is the same as we have applied in several pre-clinical studies [8,26,27]. Increasing the MI to 0.8–1.0 has in pre-clinical studies resulted in petechial bleeding (unpublished data). The MI displayed on the screen of diagnostic ultrasound scanners is based on a fixed acoustic attenuation of 0.3 dB/cm/MHz [28] and results in a conservative MI estimate. Tumors treated with FUS and MBs were located at depths ranging from 30 to 60 mm in our study. Assuming an acoustic attenuation of 0.5 dB/cm/MHz, which probably is more realistic for abdominal ultrasound, our actual *in situ* MI was 0.40–0.45.

The duty cycle used in this study was similar to what we have used in pre-clinical experiments, but the ultrasound exposure of the tumor in mice and humans is nevertheless very different. In the pre-clinical setting, we normally cover the whole tumor with one ultrasound transmit beam and apply a pulse of long duration (*e.g.,* 10000 oscillations) with a low pulse repetition frequency (*e.g.,* 0.3 Hz) to enable reperfusion of MBs. In the clinical setting, the transmit beam covers only a small part of the entire tumor, and numerous transmit beams were used to scan a given elevation slice repeatedly with a high pulse repetition frequency (*e.g.,* 3 kHz). Each elevation slice was then scanned sequentially for the total duration of treatment, and the pulse repetition frequency for sonication of a given elevation slice was 0.33 Hz. The effective duty cycle for a given part of the tumor was hence significantly lower in the clinical study than in the pre-clinical situation, where the duty cycle for the tumor is the same as the duty cycle for the probe. In a clinical situation and especially for the liver, there is significant movement between the probe and the target volume because of respiration. This is also a factor that differs from the pre-clinical situation where there is typically much less movement.

Vascularization in colorectal liver metastases takes different forms, and the angiogenetic mechanism of co-option [29] is particularly interesting in the setting of drug delivery and anti-angiogenetic therapy. Co-option involves the use of pre-existing vessels from the hosting organ, resulting in a rich supply of nutrients and oxygen. The pre-existing vessels are mature and functional, in contrast to the dysfunctional, leaky and immature vessels resulting from malignant angiogenesis. This phenomenon occurs in a significant fraction of colorectal liver metastases [30]. The abundant supply of drugs and nutrients in metastases with co-opted vessels may render a possible gain from FUS and MBs insignificant. This may also play a role in the large variability we see in response to chemotherapy and combined treatment with FUS and MBs. Poor vascularization and presence of necrosis before treatment are traits characterizing liver metastases from colorectal cancer [31]. Poor vascularization would result in low MB concentration and reduced potential biological effect of the FUS.

Timing of FUS and MB treatment in relation to chemotherapy administration is a point of optimization. Irinotecan inhibits DNA topoisomerase mainly through the active metabolite SN-38, which has a half-life ($t_{1/2}$) of approximately 10–20 h [32]. Fluorouracil disturbs synthesis of DNA and RNA as an antimetabolite to uracil, and has a $t_{½}$ of approximately 8–20 min [33]. Oxaliplatin damage DNA by formation of cross-links and has an initial $t_{½}$ of approximately 10–25 min [34,35]. A prerequisite for improved uptake of cytotoxic drugs is the simultaneous presence of biological effects in sonicated tissue and adequate levels of drugs in circulation. In this trial we intended to start treatment as soon as possible after completed infusion, but practical considerations (*e.g.,* patient relocation, navigation procedure) caused an in average 45 min time gap before the start of FUS + MBs. This means that by the time of administration of FUS + MBs, levels of oxaliplatin and fluorouracil might have been low. However, there was no correlation between volume change and the time interval between start of FUS + MB treatment and the end of the fluorouracil bolus; that is, the patients receiving MBs 30 or 60 min after chemotherapy responded similarly. To our knowledge, the persistence of potential, beneficial biological changes induced by ultrasound and MBs in the vessel wall, the ECM and the tumor cell membrane in colorectal liver metastases is unknown. However, for future trials we would recommend the application of FUS and MBs in at the same time as or immediately after chemotherapy.

The number of chemotherapy cycles before evaluation of treatment effect was limited to four in this trial. The observed tendency toward improved response in the treated group may have become more evident if the trial was prolonged with additional cycles of FUS and MBs.

The design of the present trial is unique with its strengths and shortcomings. Internal randomization of pre-selected, paired liver metastases enables efficient use of all data from included participants as they all carry their own matched control. The two study arms of lesions to be compared are similar with respect to tumor biology, genetic profile and chemotherapy regimen given, including delays and dose reductions, as they originate from the same primary tumor. However, the pre-selected lesions before randomization were heterogeneous with respect to size, shape and location in the liver.

Mixed pathologic [36] and radiologic [37] response between lesions within the same liver is a known challenge in image-based response evaluation. Tumor volume measurements from our patient cohort support this phenomenon. When considering the two pre-selected lesions before randomization, only 2 patients (12%) were mixed responders, meaning that lesions responded in opposing directions, that is, increasing and decreasing within the same liver. When considering all five lesion volumes, 10 of 17 patients (59%) were mixed responders by the same definition. Within the group of homologous responders there was also a considerable variation in degree of response, ranging from −28% to −78% as an example in one patient. A second patient responded in the range 7%–231%. Variation in tumor response is large and renders possible FUS + MB treatment-caused volume changes too small to be detected. Response evaluation with the longest diameter as used in RECIST 1.1, the standard tool used in clinical trials, makes measurement accuracy even lower. The large number of mixed responders illustrates shortcomings of the RECIST 1.1 criteria.

Adverse events recorded were similar to those commonly expected after treatment with current cytotoxic drugs. No treatment-related serious AEs occurred. One can still argue that AEs cannot be assessed directly without an external control group. However, our data strengthen the existing data supporting that treatment with the given FUS parameters is safe.

The common challenge of clinical trials in this field seems to be the small number of patients included and the large variation in tumor type, location and stage. In addition, different ultrasound parameters, MBs, study designs and endpoints are used.

A crucial step in future drug delivery studies is the development of customized dual diagnostic and therapeutic transducers optimized for therapy. This is necessary to apply the desired ultrasound parameters combined with imaging for treatment accuracy, as well as for standardization of equipment in multicenter trials. We believe that interdisciplinary cooperation is necessary for these projects to succeed. This study was performed with commercially available drugs and equipment. The

treatment is non-invasive, is safe and can be easily administered bedside to awake, cooperating patients.

Conclusion

Treatment of liver metastases from colorectal cancer with the given ultrasound settings and MB injections are safe and feasible for the patient group. Although therapeutic effect was not obtained in this trial, the technique represents a promising tool for increasing local treatment effect for cancer patients. Lack of significant therapeutic effect can be related to study design, sonication parameters applied, timing of FUS + MBs and vascular structures in the metastatic lesion. Optimal MI and duty cycle need to be studied further. In future trials, the therapeutic effect of ultrasound in drug delivery should be explored in multicenter trials. Standardized protocols for cancer type, stage, antitumor treatment, ultrasound parameters and transducers are needed to achieve useful and reproducible results. Experience from the present trial can contribute in the development of future protocols.

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Conflict of interest

The authors declare no competing interests.

Data availability statement

The research data for this article are available and can be accessed on request.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ultrasmedbio.2023.05.013.

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