Elsevier Editorial System(tm) for Journal of Controlled Release

Manuscript Draft

Manuscript Number:

Title: The release of Doxorubicin from liposomes monitored by MRI and triggered by a combination of US stimuli led to a complete tumor regression in a breast cancer mouse model

Article Type: Research paper

Keywords: Theranosis; Liposomes; Sonoporation; MRI; US-triggered drug release; Cancer

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Abstract: The work aimed at developing a novel MRI-based theranostic protocol for improving the anticancer efficacy of a Doxil-like liposomal formulation. The goal was achieved stimulating the intratumor release of the drug from the nanocarrier and favoring its diffusion in the lesion by the sequential application of low-intensity pulsed ultrasound. The protocol was tested on mice bearing a syngeneic breast cancer model. The combination of acoustic waves with different characteristics allowed for: i) the release of the drug and the co-encapsulated MRI agent (Gadoteridol) from the liposomes in the vessels of the tumor region, and ii) the extravasation of the released material, as well as intact liposomes, in the tumor stroma. The MR-T1 contrast enhancement measured in the tumor reported on the delivery and US-triggered release of Doxorubicin. The developed protocol resulted in a marked increase in the intratumor drug concentration that, in turn, led to the complete regression of the lesion. The protocol has a good clinical translatability because all the components of the theranostic agent (Doxorubicin, liposomes, Gadoteridol) are approved for human use.

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Torino, November 23rd, 2015

Dear Editor,

The manuscript entitled "*The release of Doxorubicin from liposomes monitored by MRI and triggered by a combination of US stimuli led to a complete tumor regression in a breast cancer mouse model*" authored by Silvia Rizzitelli, Pierangela Giustetto, Daniele Faletto, Daniela Delli Castelli, Silvio Aime and myself has been submitted as a research article to *Journal of Controlled Release* for your consideration.

This study represents the last part of a research started in my lab since 2011 aimed at developing a MRI-based procedure for the *in vivo* visualization of the release of an anticancer drug (Doxorubicin) from liposomes stimulated by the local application of pulsed low-intensity planar ultrasound. First, we investigated the in vitro release properties of several nanovesicular carriers under US application and demonstrated that the release was i) caused by the mechanical characteristics of the US stimulus, ii) very sensitive to the composition of the particle membrane, and iii) MRI detectable (Giustetto *et al.*, *J. Med. Imaging Health Inf.* 3, 356-366 (2013)). Next, the MRI potential of the method was demonstrated *in vivo* on a mouse model of melanoma (Rizzitelli *et al.*, *Nanomedicine* 10, 901-904 (2014)). More recently, the theranostic protocol was implemented by the encapsulation of Doxorubicin, and both the MRI performance and the therapeutic benefits associated with the US-triggered intratumor release of the drug on a mouse model of breast cancer were reported (Rizzitelli *et al.*, *J. Control. Release* 202, 21-30 (2015)).

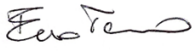
In the submitted paper, we further improved the performance of the method through the sequential application of two different low intensity US stimuli, one inducing a permeabilization of the tumor vascular endothelium, and the other stimulating the release of the drug from the nanocarrier. The synergy between the two short stimuli (only 3 minutes in total) allowed for a complete tumor regression in a mouse model of breast cancer. Furthermore, also the MRI detectability of the process was improved.

Though several examples dealing with the use of US (but mostly focused with high-intensity) for triggering drug release can be found in literature, this is, to our knowledge, the case with the highest therapeutic efficacy.

Finally, as all the components required by the protocol (low intensity US, stealth liposomes, Doxorubicin, and the MRI agent Gadoteridol) are already approved for human use, the approach has a great clinical translatability.

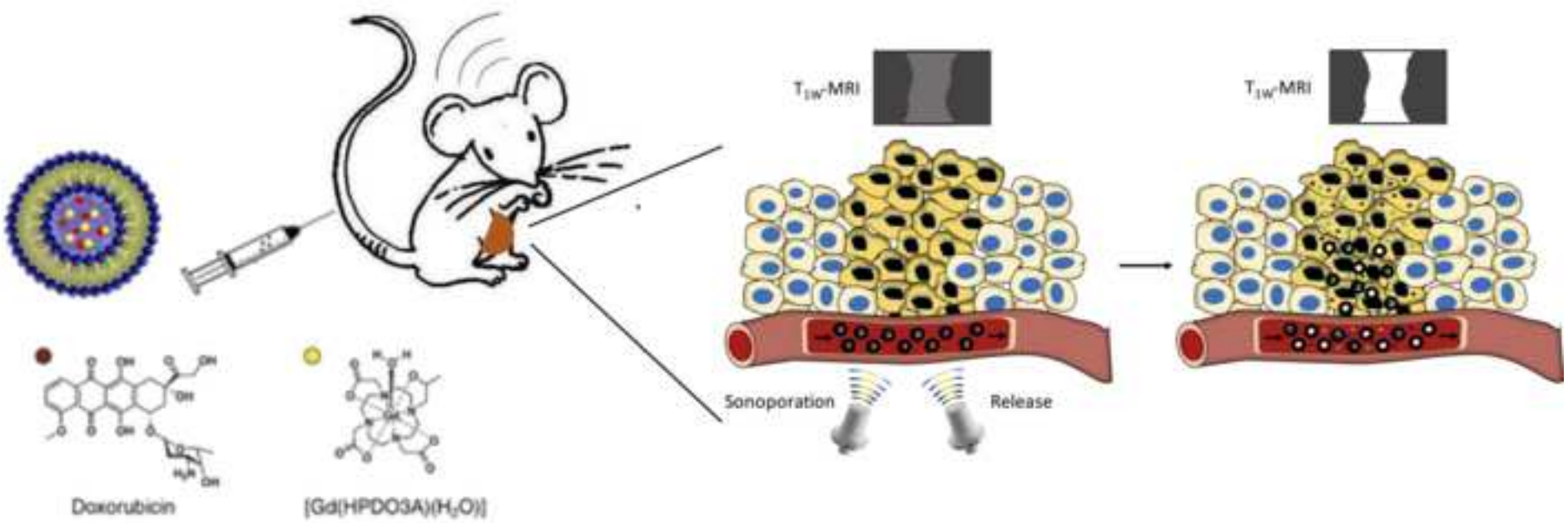
For this reason, we hope that this work might be of interest for the general audience of the Journal.

Yours sincerely,



Enzo Terreno

Graphical Abstract



The release of Doxorubicin from liposomes monitored by MRI and triggered by a combination of US stimuli led to a complete tumor regression in a breast cancer mouse model

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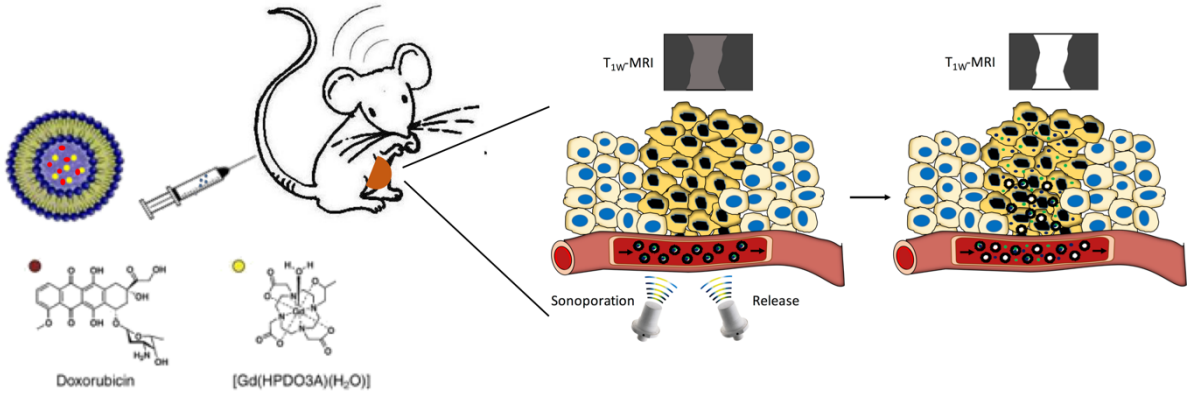
Abstract

The work aimed at developing a novel MRI-based theranostic protocol for improving the anticancer efficacy of a Doxil-like liposomal formulation. The goal was achieved stimulating the intratumor release of the drug from the nanocarrier and favoring its diffusion in the lesion by the sequential application of low-intensity pulsed ultrasound. The protocol was tested on mice bearing a syngeneic breast cancer model. The combination of acoustic waves with different characteristics allowed for: i) the release of the drug and the co-encapsulated MRI agent (Gadoteridol) from the liposomes in the vessels of the tumor region, and ii) the extravasation of the released material, as well as intact liposomes, in the tumor stroma. The MR-T_1 contrast enhancement measured in the tumor reported on the delivery and US-triggered release of Doxorubicin. The developed protocol resulted in a marked increase in the intratumor drug concentration that, in turn, led to the complete regression of the lesion. The protocol has a good clinical translatability because all the components of the theranostic agent (Doxorubicin, liposomes, Gadoteridol) are approved for human use.

Keywords

Theranosis; Liposomes; Sonoporation; MRI; US-triggered drug release; Cancer.

Graphical Abstract



1. Introduction

In recent years, much attention has been devoted to exploit the synergy between *in vivo* diagnostic imaging and drug delivery with the ultimate aim of developing improved clinical protocols for the design of therapeutic strategies on an individual basis (personalized medicine) (*1-3*). This interest has led to many studies focused to the design of *in vivo* procedures able to report on the release of a drug from its nanocarrier. Among the available imaging modalities, Magnetic Resonance Imaging (MRI) appears to be an excellent candidate because of the excellent spatio-temporal resolution, the possibility of obtaining images of deep tissues/organs, and the vast portfolio of available probes and contrast generating modalities (*4*). The use of nanocarriers as drug delivery systems has been demonstrated to yield marked improvements of the therapeutic index of the transported drug through the optimization of its accumulation at the pathological target over the other organs/tissues (*5, 6*). The nanomedicines currently approved for clinical use release the drug spontaneously, following the natural degradation of the nanocarrier that occurs when it interacts with tissue components. However, it is well established that the improvement in the control of the drug release can be achieved using specific triggers, and particular attention has been so far devoted to stimuli that lead to an increase of temperature at the pathological region (*7-12*). Besides stimulating drug release, heat may cause cytotoxic effects by itself and increase vascular permeability (*13-16*). Therefore, if the released drug is a small-sized molecule, it can freely diffuse into the tumor, thus overcoming the high interstitial pressure that usually limits the diffusion of larger nanocarrier to few cell layers beyond the vessels (*17-19*).

Recently, it has been demonstrated that liposomes can release their content upon stimulation with pulsed low intensity non-focused US (dubbed pLINFU) (*20-23*). pLINFU can be broadly defined as pulsed, planar, acoustic waves with intensity lower than 10 W/cm^2 and US frequencies ranging from low (20 kHz) to therapeutic (1-3 MHz) frequency. Differently from HIFU (High Intensity Focused US), the lower energy associated with pLINFU produce minimal or no thermal effects and the release of the drug mainly results from the mechanical interaction between the acoustic waves and the nanocarrier. Non thermal release may have some advantages over heating such as: i) providing the access to the stimulated drug release to non temperature-sensitive carriers, ii) making not longer necessary the control of the local

temperature, and iii) shortening the stimulation time. Drug release from nanocarriers upon pLINFU exposure has been demonstrated both *in vitro* and *in vivo* (*24-27*).

The possibility of monitoring *in vivo* and in real-time the effective release of the drug is of paramount importance, and MRI has been demonstrated an excellent technique to achieve this scope. A practical approach to visualize the release of the drug from liposomes consists of encapsulating a hydrophilic paramagnetic agent (based on Gd^{3+} or Mn^{2+} ions) in the aqueous inner cavity of the nanovesicle. Upon the entrapment, the MRI contrast is "silenced" and its activity is recovered when the agent is released (*10, 23, 28-32*).

We proved the *in vivo* feasibility of this approach to monitor the pLINFU-triggered release of the clinically approved MRI agent Gadoteridol (Chart 1, left) from a sterically stabilized liposomal formulation (*27*). More recently, we successfully applied this protocol to demonstrate the ability of Gadoteridol to act as an *in vivo* imaging reporter of the release of Doxorubicin from a Doxil-like formulation (*32*). Even more important, we observed that the local application of pLINFU significantly ameliorated the therapeutic outcome of the liposomal drug in a murine model of breast cancer.

Herein, we report an implementation of this method aimed at further improving the therapeutic efficacy. The proposed methodology relies on the local sequential application of two different pulsed US: one, herein termed "release stimulus", designed to trigger the release of the drug from the liposomes circulating in the tumor vasculature, and the other, termed "sonoporation stimulus", applied to increase the tumor vascular permeability in order to favor the diffusion of the drug in the tumor stroma. Sonoporation is already widely used in drug-delivery for increasing the permeability of vascular endothelium through a proper combination of US and nano/microparticles (*33, 34*).

2. Materials and Methods

2.1 Chemicals

1,2-Dipalmitoyl-sn-glycero-3-phosphocoline(DPPC), 1,2-Distearoyl-sn-glycero-3-phosphocoline (DSPC), Cholesterol (Chol), and 1,2 Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]Ammonium salt (DSPE-PEG2000) were purchased from Avanti Polar Inc. (Alabaster, AL, USA). Doxorubicin hydrochloride (as a powder) was purchased from Sigma. Gadoteridol (Gd-HPDO3A) was kindly provided by Bracco Imaging (Colleretto Giacosa (TO), Italy). Gd-DOTAMA$(C18)_2$ complex was

synthetized according to the published procedure (*34*). The culture medium RPMI 1640, biological buffers, fetal bovine serum (FBS), glutamine, penicillin-streptomycin mixture, and trypsin were purchased from Cambrex (East Rutherford, NJ, USA).

2.2 Liposomes preparation

Doxo-Gado-Lipo samples were formulated with DPPC/DSPC/Chol/DSPE-PEG2000 (10:5:4:1 molar ratio) and prepared according to the method reported in ref. *31*.

Gd_{INC}-LIPO sample was composed by DPPC/DSPC/Chol/Gd-DOTAMA$(C18)_2$/DSPE-PEG2000 (8:3:4:4:1 molar ratio) and did not contain neither Doxorubicin nor Gadoteridol. The lipid film was hydrated with HEPES buffer.

2.3 Liposomes characterization

The mean hydrodynamic diameter of the liposomes was determined (three replicates) by dynamic light scattering (Malvern ZS Nanosizer, Malvern Instrumentation, UK). The measurements were carried out at 25°C with a scattering angle of 90°C. Polydispersion Index (PDI) was taken to indicate the width of the particle size distribution.

The concentration of the loaded Gadoteridol was determined relaxometrically (Stelar Spinmaster, Mede (PV), Italy) measuring the T_1 of the water protons at 21 MHz and 25°C after the complete degradation of liposomes and demetallation of Gadoteridol upon addition of hydrochloric acid and heating at 180°C overnight. The amount of loaded Doxorubicin was determined spectrofluorimetrically (λ_{abs} = 488 nm, λ_{em} = 590 nm) after complete releasing from liposomes obtained adding the surfactant Triton X-100. The molar ratio between encapsulated Gadoteridol and Doxorubicin was 10.9:1.

2.4 US apparatus

"Release" stimulus was applied using a 3 MHz (±100 kHz) custom US transducer. At 37 V of alimentation tension, the Spatial Average Pulse Average Intensity (I^{SAPA}) over the beam cross-sectional area was 5.4 ± 0.2 W/cm^2, corresponding to an Acoustic Pressure of 0.28 MPa (Mechanical Index of 0.18). The Spatial Average Temporal Average Intensity (I^{SATA}) value was 2.8 ± 0.1 W/cm^2. The total insonation time was 2 minutes, with a duty cycle of 50% and a Pulse Repetition Frequency (PRF) of 4 Hz.

The "sonoporation" stimulus was applied using a 1 MHz (± 100 kHz) custom US transducer. The alimentation tension was set to 19 V, which yielded an I^{SAPA} value over the beam cross-sectional area of 6.5 ± 0.3 W/cm^2, corresponding to an Acoustic Pressure of 0.15

MPa (Mechanical Index of 0.11). The ISATA value was 0.78 ± 0.02 W/cm^2. The total insonation time was 1 minute, with a duty cycle of 12%, and a PRF of 1 Hz.

Both the ultrasound transducers were designed and realized in collaboration with TEMAT s.r.l. (Torino, Italy). Piezoelectric ceramic flat disc transducer (STEMiNC Steiner & Martins, Inc, USA), 25 mm of diameter, was connected to a specific oscillator driving circuit for the generation of the ultrasound energy in all the experiments. The circuit included a tension generator (TTi EX354 RD, dual power supply 280 W) and a waveform generator (LXI KEITHLEY 3390, 50 MHz). The performances of US transducer were controlled using an oscilloscope voltage signal (Tektronix TDS1001B) with an attenuated (100x) oscilloscope probe for the connection to the circuit, and multimeter (Fluke 87 V) for the current drawn. The multimeter was inserted in series between the power generator and the oscillating circuit to monitor current absorption from operating piezoelectric component and oscilloscope (TEKTRONIX TDS 1001 B – two channel – 40 MHz 500 MS/s) at oscillatory output point to evaluate sinusoidal voltage amplitude. The piezoelectric disk was housed and fixed inside a cylinder (metal alloy ultrasound transmitter) made by two round concentric chambers. The disc was cooled down with water circulating in the external chamber. The cooling system was turned on during all the insonation time.

2.5 Experimental in vivo US setup

The 1 MHz and 3 MHz US transducers described above were used for the *in vivo* experiments. A three-layer interface composed by ultrasound gel, agar layer (1 mm) and ultrasound gel was positioned between the 1 MHz transducer and the tumors, whereas a multi-layered interface composed by ultrasound hydro gel, agar gel, castor oil, mineral oil and silicon oil (from the tumor to the transducer) was used for the application of the release stimulus (*32*). This multi-layered compound is composed of overlapping layers deposited on the tumor's surface to obtain uniform and separated interface layers with the aim of decreasing the intensity backscattering and so increasing the intensity on the tumor target the composition of these layers is made with a decreasing value of acoustic impedance, from tumor to transducer. This system interface was optimized in previous experiments.

2.6 Cells

TS/A cell line derives from a spontaneous mammary adenocarcinoma that arose in a retired breeder BALB/c female (*35*). Cells were cultured as monolayer at 37°C in a 5% CO_2-

containing humidified atmosphere and RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

2.7 Animal model

Female BALB/c mice, 6 weeks-old, were purchased by Charles River Laboratories and kept in standard housing (12 h light-dark cycles) with a standard rodent chow and water available *ad libitum*. Experiments were performed according to the national regulations and were approved by the local animal experiments ethical committee. To induce mammary adenocarcinomas, $6x10^5$ TS/A cells were inoculated subcutaneously in the abdominal wall of a mouse. The experimental protocols (see below) started one week from the cells inoculation, when the tumor reached a volume of 50±10 mm^3. Tumor volume was determined by MRI (multislice T_{2w} images). Mice were anesthetized by intramuscular injection of tielamine/zolazepam (Zoletil®) 20 mg/kg bw and xylazine (Rompum®) 5 mg/kg bw.

2.8 MRI measurements

In vivo MRI experiments were performed on a Bruker Avance 300 MHz equipped with a microimaging (2.5 Micro) probe. The MRI session consisted of a series of T_1-weighted (pulse sequence: Multi Slice Multi Echo, TR 250 ms, TE 3.2 ms, 6 averages, acquisitions time 3.12 min, matrix size 128x128, slice thickness 1 mm, FOV 3x3 cm) and T_2-weighted images (pulse sequence: Rapid Acquisition with Refocused Echoes, TR 2000 ms, TE 3.4 ms, effective TE 27.20 ms, 4 averages, acquisition time 1.04 min, matrix size 128x128, slice thickness 1 mm, FOV 3x3 cm). MR T_{1w} and T_{2w} images were acquired before the liposome injection (pre-contrast) and then, after the US applications, within the first hour, after 6 hours and daily until 7 days. Liposomes injection, as well MRI analysis, was repeated once a week for three weeks. T_{2w} images were acquired to define the morphology and draw the ROI (Region of Interest) around the tumor. ROIs were then translated on T_{1w} images, and the T_1-Contrast-to-Noise Ratio (T_1-CNR) was measured as follows:

$$T_1 - CNR = \frac{SI_{(A)} - SI_{(B)}}{SDV_{(B)}}$$

$SI_{(A)}$ is the signal intensity of the given ROI, $SI_{(B)}$ is the signal of the background, and $SDV_{(B)}$ is the standard deviation of the signal noise.

The values reported in the graphs are expressed as T_1-CNR%, which compares the T_1-CNR values measured pre- and post-injection of paramagnetic liposomes:

$$T_1 - CNR\% = \frac{CNR_{(Post)} - CNR_{(Pre)}}{CNR_{(Pre)}} \times 100$$

2.9 Experimental scheme

The following groups of mice were enrolled in the study:

A) pLINFU group (3 mice administered with Doxo-Gado-Lipo and subjected to the release US pulse)
B) SONO group (3 mice administered with Doxo-Gado-Lipo that received the sonoporation stimulus at the time of the administration of the theranostic agent)
C) SONO-pLINFU group (3 mice administered with Doxo-Gado-Lipo that received the sequential, B+A, US stimuli),
D) Control group (3 mice injected with Doxo-Gado-Lipo that did not receive any US stimulation).
E) CTRL no LIPO (3 mice not injected with Doxo-Gado-Lipo that did not receive any US stimulation). This group was only used for the assessment of the therapeutic efficacy.

Doxo-Gado-Lipo were injected at a Gd dose of 0.1 mmol/kg bw and at a Doxorubicin dose of 5 mg/kg bw. For the experiments with Gd_{INC}-Lipo, the dose of injected Gd was 0.09 mmol/kg bw.

The tumor volume of each mouse was monitored by MRI until 19 days from the beginning of the study. The contrast enhancement in the tumor was measured for one hour consecutively after the treatment.

All animals were anesthetized 15 min before the treatment. Before each MRI check, animals were weighed and the body temperature was acquired in order to avoid and monitor possible changes due to side effects of the drug.

Before the experiment, tumors were shaved to minimize US reflection. For the same reason, during US application, mice were placed on an agar cot (2 cm thick) and covered with a surgical drape with the exception of the tumor.

Mice belonging to the SONO-pLINFU group were subjected to a sonoporation stimulus during the injection of liposomes and immediately after, to a pLINFU shot. Next, MRI scans were acquired in the first hour after liposomes injection to measure both T_1-CNR % and monitoring tumor progression.

2.10 Determination of intratumor Gd content

The amount of Gd in the explanted tumors was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Element-2; Thermo-Finnigan, Rodano (MI), Italy). After the treatment, mice of the groups that received Gadoteridol (pLINFU, SONO, SONO-pLINFU, and Control groups) were sacrificed by cervical dislocation. Tumors were gently removed and sample digestion was performed with 1.5 ml of concentrated HNO_3 (70%) under microwawe heating (Milestone MicroSYNTH Microwave labstation equipped with an optical fiber temperature control and HPR-1000/6M six position high-pressure reactor, Bergamo, Italy). After digestion, the volume of each sample was brought to 3 mL with ultrapure water and the sample was analyzed by ICP-MS. Three replicates of each sample solution were analyzed.

2.11 Statistical analysis

The mean signal intensity values were calculated in regions of interest (ROIs) manually drawn on tumors. Analysis of images was performed in Bruker ParaVision, version 5.1. ROIs were manually defined by drawing contours around the tumor area in every image slice and T_2-weighted images served as reference. All data are expressed as mean value ± standard error (SE).

3. Results

3.1 The MRI contrast detected in the tumor reports on the intratumor amount of drug

All the mice enrolled in the study were systemically injected with sterically stabilized liposomes loaded with Doxorubicin and Gadoteridol (dubbed Doxo-Gado-Lipo), formulated to mimic the clinically approved chemotherapeutic Doxil®. Liposomes displayed a hydrodynamic diameter of 150 nm with a PDI lower than 0.1 as determined by Dynamic Laser Scattering measurements.

Four animal groups (three mice each) were enrolled in the study: A) the pLINFU group (mice administered with Doxo-Gado-Lipo and subjected to the release US pulse), B) the SONO group (mice administered with Doxo-Gado-Lipo that received the sonoporation stimulus at the time of the administration of the theranostic agent), C) the SONO-pLINFU group (mice administered with Doxo-Gado-Lipo that received the sequential, B+A, US stimuli), and D) the control group (mice injected with Doxo-Gado-Lipo that did not receive any US stimulation).

To get information on the *in vivo* release of the liposomal content after the US stimulation, the T_1-MRI contrast arising from the paramagnetic agent Gadoteridol was measured in the tumor region within the first hour after the injection of the liposomes. The contrast was expressed as percentage change in the Contrast-to-Noise ratio (T_1-CNR%, see Material and Methods).

The results obtained are illustrated in Figure 1. The T_1-CNR% values measured for both the treated animals (pLINFU and SONO-pLINFU groups) were *ca.* 250 % and 120% higher than the control group, respectively. Conversely, the corresponding values measured for the SONO group were similar (in absolute values) to the control, but negative. This observation suggests that the single sonoporation stimulus induced an effect, but, more likely, not associable with the release of Gadoteridol (and consequently of Doxorubicin) as already observed *in vitro*.

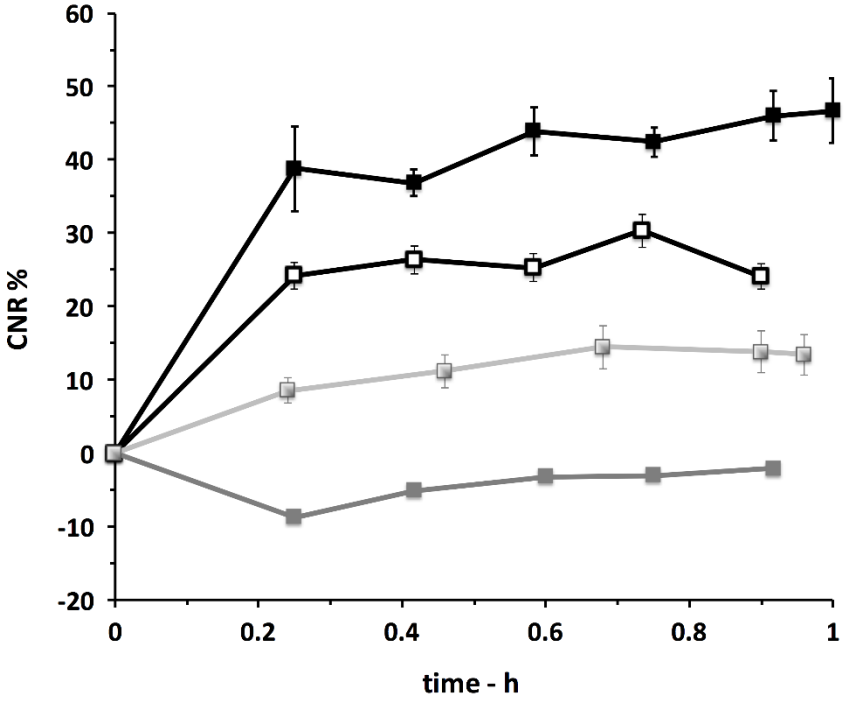


Fig 1. MRI contrast in the tumor after injection of Doxo-Gado-Lipo. Time evolution of T_1-CNR% after administration of Doxo-Gado-Lipo (dose of 0.1 mmol/kg bw of Gadoteridol and 5 mg/kg bw of Doxorubicin). Black: SONO-pLINFU group; white: pLINFU group; light grey: control group; dark grey: SONO group. Mean ± SE is reported, n = 3 per group.

The larger contrast enhancement observed for the SONO-pLINFU group with respect to the pLINFU group suggests the presence of a higher amount of Gadoteridol in the tumor of the mice that received the combined insonation.

To confirm this hypothesis, we measured the total amount of Gd in the explanted tumors of the four groups of animals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Figure 2 clearly shows that the tumors undergone to the combination of acoustic waves contained a higher amount of Gd than the specimens of the animals that received a single stimulus, and much higher than the control group. As we did not observe (*in vitro*) any release of Gadoteridol from the liposomes that received the sonoporation stimulus only, the observation of an increased amount of Gd in the tumors of the SONO group supports the view that the sonoporation pulse caused the extravasation of intact liposomes.

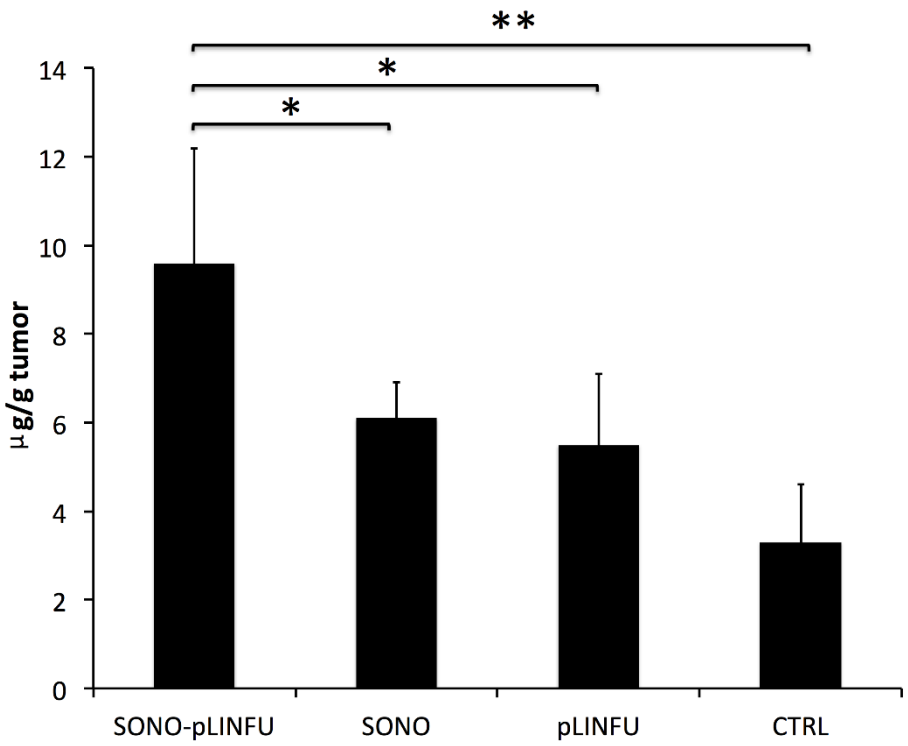


Fig 2. Amount of Gadolinium in explanted tumors. Amount of Gd, normalized to the tumor weight, measured by ICP-MS in the tumors of the different groups of mice excised 1 hour after the administration of Doxo-Gado-Lipo. Mean ± SE is reported, (*) $p < 0.05$, (**) $p < 0.01$, n = 3 per group.

To test this hypothesis, liposomes (coded Gd_{INC}-Lipo) incorporating the amphiphilic complex Gd-DOTAMA$(C_{18})_2$ (*36*) (Chart 1, right) in their membranes were prepared (hydrodynamic diameter 145 nm, PDI < 0.1) on the consideration that amphi/lipo-philic

systems are much less prone to be released from liposomes than water soluble ones. It follows that the contrast enhancement measured in the tumor region after the systemic injection of Gd_{INC}-Lipo will report on the amount of intact liposomes (circulating + extravasating) in the tumor lesion.

In this experiment, two groups of mice were systemically administered with Gd_{INC}-Lipo. Next, one group was treated with the sonoporation pulse, whereas the other was not and acted as control. As done in the previous experiments, the two groups were subjected to MRI and the T_1-CNR% was observed for 1 hour after the liposomes injection. The tumors of the treated group showed a higher contrast enhancement than the control (Figure 3), thereby supporting the initial hypothesis that the sonoporation stimulus caused the extravasation of intact liposomes. The lower T_1CNR% values measured for the control group can be attributed to the liposomes circulating in the tumor vasculature.

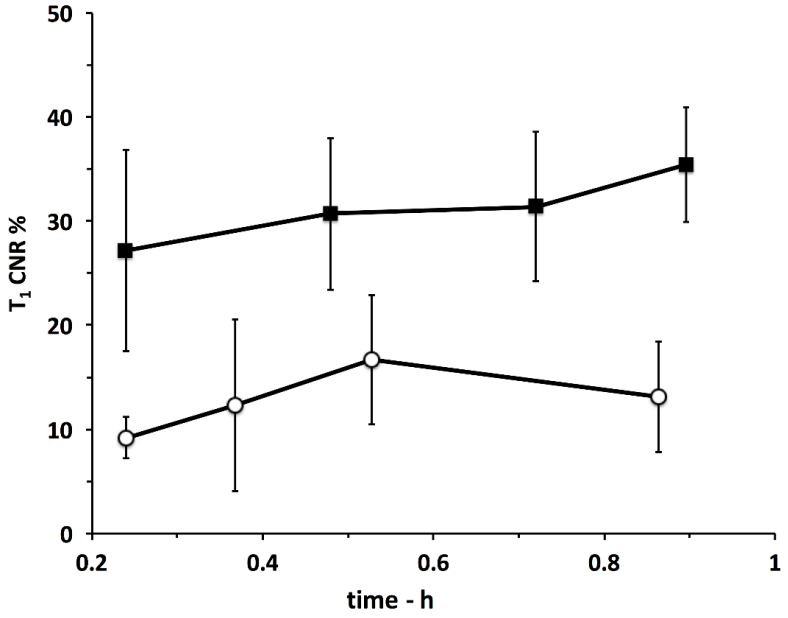


Fig. 3. Tumor MRI contrast after injection of Gd_{INC}-Lipo. MRI Temporal evolution of T_1-CNR% measured in the tumor after the administration of Gd_{INC}-Lipo (0.09 mmol Gd/kg bw) with (black squares) or without (white circles) the application of the sonoporation pulse. Mean ± SE is reported, n = 3 per group.

3.3 The combined use of US led to a complete tumor regression

The therapeutic efficacy of the proposed protocol on a syngeneic mouse model of breast cancer was investigated. Mice were systemically administered with Doxo-Gado-Lipo (dose of Doxorubicin 5 mg/kg bw) at day 0, 7, and 14. An additional control group (CTRL no LIPO) was included in the study in which the animals were not treated with the theranostic nanomedicine. The animals were recruited in the study (day 0) when the tumor volume was 50 ± 10 mm^3. The therapeutic efficacy of the treatment was monitored by acquiring multislice anatomical T_{2W} MR Images that allowed for an accurate measurement of the tumor volume.

As illustrated in Figure 4, after three weeks of treatment the tumor size (normalized to the volume at day 0) of the experimental groups followed the order: Control > pLINFU > SONO > SONO-pLINFU (the mice of the control group were sacrificed after two weeks of treatment for ethical reasons). Impressively, the tumor of the mice of the SONO-pLINFU group regressed almost completely. The tumors of mice only stimulated with sonoporation did not grown during the three weeks of treatment, whereas those ones of the mice exposed to pLINFU displayed larger volumes than SONO group, but smaller than the Control group and much smaller than the group not treated with the drug.

Collectively, these results suggest that sonoporation have a dominant effect in the tumor regression observed for the mice treated with the sonoporation/pLINFU combination.

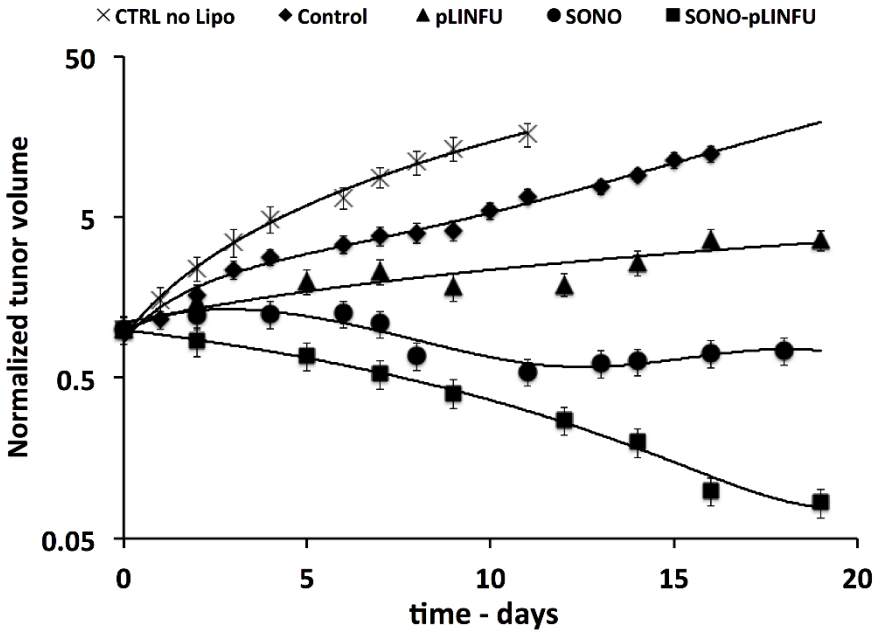


Fig. 4. Tumor progression monitored by MRI. Semi-logarithmic plot reporting the normalized tumor growth ($Volume_t/Volume_0$) as assessed by $T_{2\text{-weighted}}$ MR images. Sonoporation was applied during the liposomes injection, while pLINFU was applied just after sonoporation.

CTRL no LIPO refers to a group of mice that was not injected with liposomes. Mean ± SE is reported, n = 3 per group.

Figure 5 shows a panel of MR images to appreciate the effect on the tumor growth caused by the different US-based stimulations.

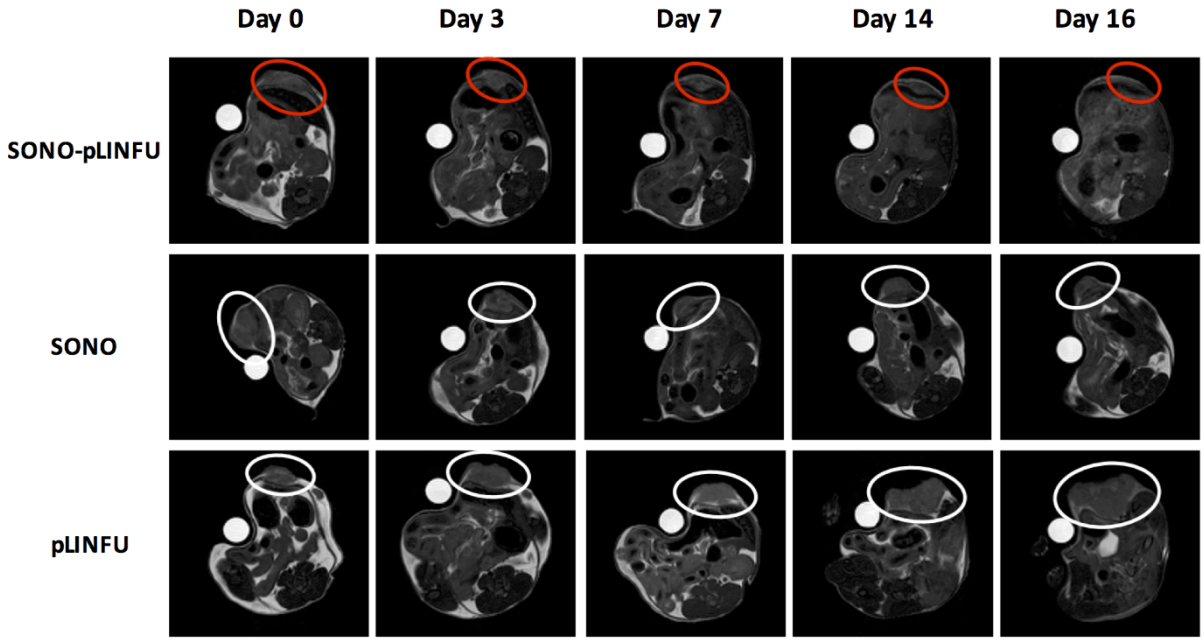


Fig. 5. Representative MR images of the US-treated mice. Representative T_1-weighted axial MR images (7 T) of mice belonging to the groups indicated on the left. The images were acquired at the time of the first MRI session (day 0), and then at days 3, 7, 14, and 16.

4. Discussion

The main aim of this work was to improve the therapeutic output of a theranostic protocol based on the MRI visualization of the release of Doxorubicin from liposomes stimulated by a combination of US pulses able to trigger the release of the drug from the carrier and to increases the tumor vascular permeability, thus favoring the overall intratumor diffusion of the drug in the tumor.

We have been already demonstrated that the local application of pLINFU can stimulate the drug release from the inner cavity of liposomes primarily through non-thermal effects (*10, 23, 32*). On the other hand, sonoporation has been widely used to favor the extravasation of drugs or other bioactive compounds from blood circulation, thus promoting its accumulation at the pathological target site.

Typically, sonoporation is induced by the inertial cavitation of microbubbles (in some cases also liposomes (*37*)) occurring upon application of high intensity US waves. However, it has been reported that also stable cavitation of microbubbles induced by low intensity US pulses can increase cell membrane permeability (*38, 39*).

A low acoustic pressure is necessary to induce sonoporation and several mechanisms have been proposed to explain the increased permeability including shear stress and enhanced endocytosis. It is important to point out that, differently from the effect induced by pLINFU, the characteristics of the sonoporation pulse used in this work were not able to trigger the release of the liposomal content *in vitro*. On the other hand, it is worth noting that we have recently observed that the interaction between pLINFU and liposomes can increase the permeabilization of the tumor vascularization (*32*).

In this very complex scenario, we surmised that the combination of US stimuli able to both trigger drug release and enhance vascular permeability could boost the accumulation and intratumor diffusion of the drug (as well as the MRI agent) transported by the liposomes, with consequent benefits for imaging detection and especially for the therapeutic outcome. To achieve this task, it is fundamental that the stimuli are applied when the intratumor concentration of the nanomedicine is maximum, *i.e.* just after the systemic administration of the liposomes. In fact, though endowed with stealth properties towards the immune system, the injected nanomedicine is quite rapidly taken up by liver and spleen, thus reducing the amount of circulating drug available for the intratumor release. Furthermore, since it has been observed that the sonoporation-induced opening and resealing of biological membranes may take from seconds to hours (*38*), we decided to apply the “sonoporation” stimulus (duration 1 min) at the time of the injection of the liposomes, *i.e.* just before the application of the US triggering the drug release (duration 2 min). The rational was to first create pores in the tumor vasculature and immediately after induce the release and intratumor diffusion of the drug.

Interestingly, the T_1-MRI contrast enhancement measured in the tumor region of mice undergone to both US stimuli was higher than the corresponding value obtained for the mice

that received only the release stimulation (Figure 1). Since the contrast is a direct measure of the amount of the MRI agent (Gadoteridol) in the region of interest, this result suggests that the sonoporation stimulus increased the total amount of the MRI probe in the tumor. This finding was supported by *ex-vivo* ICP-MS analysis carried out on tumors specimens (Figure 2), which confirmed the higher amount of Gadolinium in the tumors of mice treated with the combination of US stimuli with respect to the lesions of the animals exposed to the release stimulation only. Moreover, such measurements showed that a good amount of contrast agent (similar to the value measured upon application of the release shot) was present in the tumor tissue of mice that were treated only with the sonoporation stimulus. Since this latter stimulation was unable to trigger the release of Gadoteridol from liposomes, it follows that the increased intratumor amount of the MRI agent induced by the sonoporation treatment can be justified by the blood extravasation of intact liposomes. Consistent with this view is the negative contrast enhancement measured in the tumors of the SONO group (Figure 1), which can be the result of the detrimental (to T_1 contrast) T_2 shortening effect associated with the presence of intact paramagnetic liposomes, which is expected to be stronger at high magnetic fields (*40,41*). To further support this hypothesis, liposomes incorporating an amphiphilic Gd-complex (Gd-DOTAMA(C18)$_2$, Chart 1) in the bilayer were prepared and injected in mice of the SONO group. The MRI contrast arising from this kind of agent allows for a much more accurate measurement of the amount of intact liposomes due to the higher loading stability of the paramagnetic payload when embedded in the membrane. The T_1 contrast measured in the tumor exposed to the "sonoporation" stimulus was much higher (two/three fold) than the corresponding values obtained for the control group (Figure 3), thus further reinforcing the view that the pores created by the sonoporation stimulus on the tumor vascular endothelium allowed for the blood extravasation of intact liposomes.

The mechanism underlying this process is still unclear. One hypothesis is that, in analogy to what reported in literature, liposomes hit by low intensity US might propagate the energy associated with the acoustic waves in adjacent tissues making them oscillating/vibrating. It has been reported that these oscillations led to transient pore formation and concomitant transient intracellular calcium entrance and hydrogen peroxide production that facilitate endocytosis process (*42, 43*). These effects can modify vascular permeability, thus promoting the extravasation of liposomes. However, also shear stress and

radiation acoustic forces processes might be involved in the extravasation of the nanoparticles.

As it has been demonstrated that Gadoteridol and Doxorubicin share the same release properties upon application of the release stimulus (*32*), the amount of Gadoteridol found in the tumor directly reports on the amount of the drug as well. On this basis, it is expected that the therapeutic effect shows a good correlation with the quantitative data displayed in Figure 2. Actually, the progression of the tumor growth reported in Figure 4 confirmed this anticipation. 11 days after the enrollment of the mice in the study, the tumors of the mice that did not received the liposomal drug displayed a tumor volume *ca.* 16 times larger than the value at day 0. After the same time, the lesions of the animals that received the drug, but without any US stimulation, decreased of a factor of 2.5, those received the "release" stimulus of a factor of 6.3, those exposed to the "sonoporation" shot of a factor of 30.4, and those exposed to the combination of stimuli decreased of a factor of 55. These differences further increased at longer times especially for SONO and SONO-pLINFU groups.

Importantly, the synergic effect of the combined local application of the two US stimuli yielded an impressive therapeutic benefit on this breast tumor model with an almost complete regression of the lesion after three administration of the drug and a total US stimulation time of only 9 min (3 min of sonoporation and 6 min of pLINFU). It is noteworthy that in the therapeutic procedure involving the thermal release of Doxorubicin from liposomes, the heat stimulus is applied for much longer times (*44*). Very few examples of a tumor regression like that reported here can be found in the literature for Doxorubicin-loaded liposomes exposed to stimuli for triggering the release of the drug (*45,46*), and, to our knowledge, none used low intensity US stimulation.

5. Conclusions

In conclusion, an innovative MRI guided theranostic protocol for the visualization of the intratumor release of Doxorubicin from stealth liposomes stimulated by the sequential combination of planar low intensity US to release the drug from the carrier and favoring its diffusion in the tumor has been successfully tested on a syngeneic breast cancer model on mice. Such a combination was designed to create a synergy between the increase the permeabilization of the tumor vascular endothelium induced by a "sonoporation" stimulus and the release of the drug produced by a different US stimulation. This approach significantly

improved the therapeutic effect of the drug with an almost complete regression of the tumor after three weeks of treatment and a total of 9 min of US stimulation. The overall procedure was monitored by MRI, which allowed for an *in vivo* quantification of the drug in the lesion, the assessment of the effectiveness of the US-triggered drug release, and the monitoring of the therapeutic efficacy. Though the performance of the protocol needs to be tested in non superficial tumor models, the theranostic agent here investigated appears to be particularly promising for the clinical translation of the protocol because it is based on a liposomal formulation very similar to those (*e.g.* Doxil®) already approved for human use, implemented with the encapsulation of the clinically used MRI agent Gadoteridol (marketed as ProHance™).

6. Acknowledgements

The work was supported by University of Torino/Compagnia San Paolo (project "Innovative Nanosized Theranostic Agents"). The support from the EU-COST Action TD1004 (Theragnostics Imaging and Therapy) is gratefully acknowledged.

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