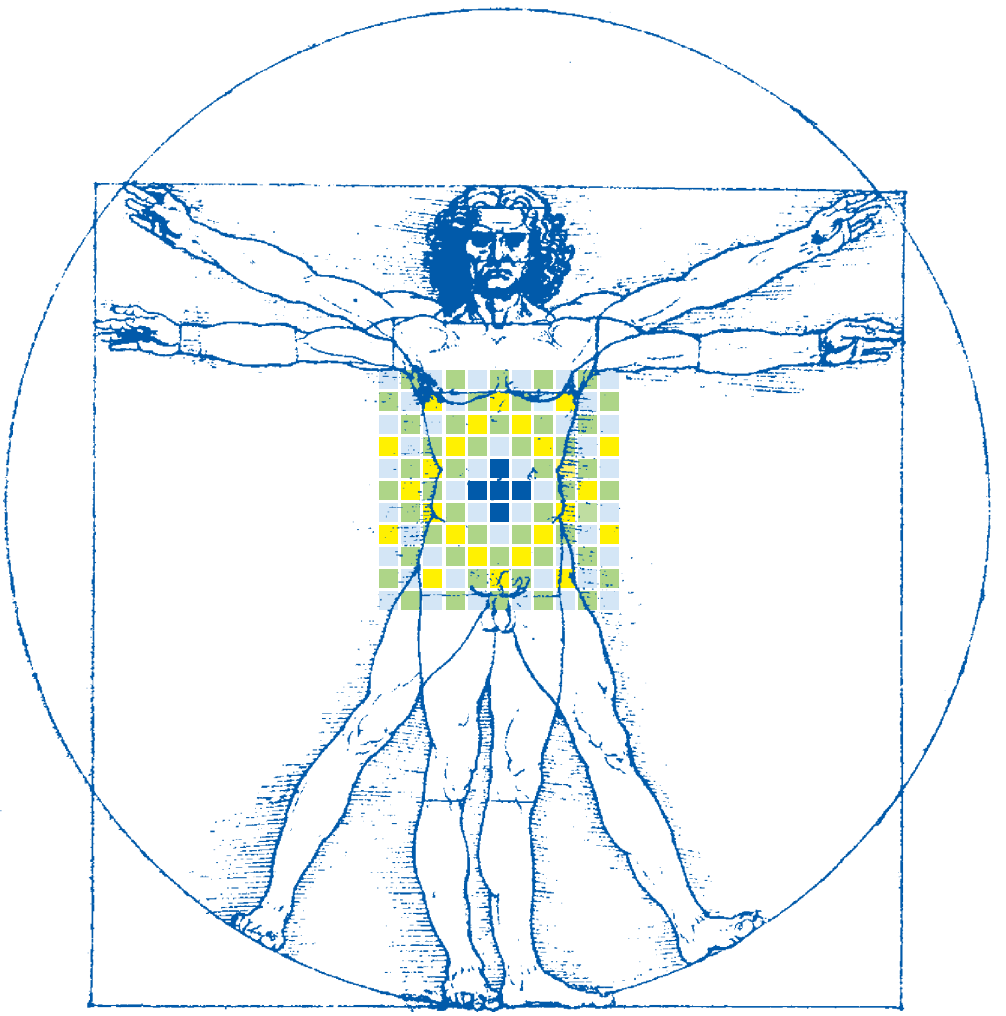
THE QUARTERLY JOURNAL
OF
NUCLEAR MEDICINE
AND
MOLECULAR IMAGING

AFFILIATED TO
THE SOCIETY OF RADIOPHARMACEUTICAL SCIENCES
IE INTERNATIONAL RESEARCH GROUP OF IMMUNOSCINTIGRAPHY AND THERAPY (IRIST)

OLUME 61 - No. 1 - MARCH 2017



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The Quarterly Journal of Nuclear Medicine and Molecular Imaging 2017 March;61(1):76-94
DOI: 10.23736/S1824-4785.16.02944-7

REVIEW

PRECLINICAL IMAGING

Advances in molecular preclinical therapy mediated by imaging

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ABSTRACT

Several advances have been made toward understanding the biology of cancer and most of them are due to robust genetic studies that led to the scientific recognition that although many patients have the same type of cancer their tumors may have harbored different molecular alterations. Personalized therapy and the development of advanced techniques of preclinical imaging and new murine models of disease are emerging concepts that are allowing mapping of disease markers *in vivo* and in some cases also receptor targeted therapy. Aim of this review is to illustrate some emerging models of disease that allow patient tumor implantation in mice for subsequent drug testing and advanced approaches for therapy mediated by preclinical imaging. In particular we discuss targeted therapy mediated by high frequency ultrasound and magnetic resonance, two emerging techniques in molecular preclinical therapy.

(*Cite this article as:* Greco A, Albanese S, Auletta L, De Carlo F, Salvatore M, Howard CM, *et al.* Advances in molecular preclinical therapy mediated by imaging. Q J Nucl Med Mol Imaging 2017;61:76-94. DOI: 10.23736/S1824-4785.16.02944-7)

Key words: Molecular imaging - Magnetic resonance imaging - Ultrasonography - Gene therapy - Molecular targeted therapy.

In the last years several advances have been made toward understanding the biology of cancer. In particular it has been observed that although many patients have the same type of cancer their tumors harbor different molecular alterations. This has led to the concept of personalized therapy and the development of advanced techniques of preclinical imaging and new mouse models of disease. Preclinical imaging allows mapping of disease markers *in vivo* and in some cases also receptor targeted therapy.

At the same time mouse models of disease represent a direct link from *in vitro* studies to clinical trial in patients. Mice have a well-characterized genetic pattern, which is very similar to that of humans; human and mouse genomes are approximately 85% identical. Furthermore, mice are easy to reproduce and to maintain and relatively inexpensive to breed. However, in some cases, mouse models have failed to be predictive.[1] For example rodent models, correctly predicted human toxicity in only 43% of the cases when considering a big comparative study to test the concordance between toxicity of pharmaceuticals in humans and animals.[2] The maximum tolerated dose (MTD) of drugs in mice is higher than in humans, so it was be necessary to evaluate drugs by using doses comparable to patients. Moreover, despite successful preclinical testing, only 15% of

early clinical trial succeed and also very few drugs are approved for clinical study by the Food and Drug Administrations (FDA). On the other side, the efficiency of translational models could be increased when used together with emerging translational approaches like advanced preclinical molecular techniques. The aim of this review was to illustrate some emerging models of disease that allow patient tumor implantation in mice for subsequent drug testing and advanced approaches for therapy mediated by preclinical imaging. In particular we will describe targeted therapy mediated by high frequency ultrasound and magnetic resonance, two emerging techniques in molecular preclinical therapy.

Mice models for personalized cancer treatment

A thorough study of the human genome allows the characterization and separation of patients based on their genetic defects and the classification of subgroups of tumors that have the best chance of survival based on several prognostic factors. According to that, patients can be stratified between those who respond better to one therapy rather than to another. Because it is not always easy to perform human clinical trials based on cost and difficulties in patient recruitment, a new reality is emerging consisting of co-clinical trials performed at the same time on patients and on murine models of disease.

Currently, there are two personalized mouse models of disease: the genetically engineered mouse models (GEMM) of cancer and patient derived tumor xenograft models (PDX). These models are now used in most hospital for co-clinical trials to guide therapy in ongoing human patient trials.

GEMM mouse models used in co-clinical trial

GEMM models are used as part of phase I/II trials in humans for drug development.[3, 4] During these trials there is a comparison and integration between data obtained both in mouse and humans. The identification of the genetic basis of therapeutic response obtained in mouse models is important for patient stratification in clinical trial. The concept is based on GEMM cancer mouse models in which the genetic profile is modified so that one or more genes that are involved in the transformation process or malignancy are mutated, deleted or overexpressed. This method allows the study of the effects of these genetic alterations over time and to assess the therapeutic response of these tumors enabling the identification of the genetic basis of therapeutic response. One of the advantages of these models consists of the fact that these animals have an intact immune system. Several anticancer therapies today are based on the stimulation of the immune system of patients, leading their immune system to eradicate tumors.[5] Also an accurate GEMM model, compared with xenografts models often mimics much better human cancer histologically. This structural difference of the tumor, affects the results of drug therapies, and also the effects of response to therapy.[6] In these models also, it is possible to study the stage of the disease and to test different drugs at different stages of the tumor development. Mouse models can often be expanded to more subjects in which it is possible to test different pharmaceutical compounds.

However, GEMM models also have several disadvantages: usually it is difficult to drive the extensive genetic alterations that occur in human cancer. Heterogeneity can exist also within the same tumor, and often GEMM models show heterogeneity in the same cancer model. These facts do not help developing targeted therapies, and often researchers find effective antitumor molecules in mouse models that do not translate efficiently in human settings. Furthermore, the development of GEMM models often requires longer time periods, and not all mice at the end develop pathological features of the tumor disease. For example among the $TR\beta^{PV/PV}$ mouse models of follicular thyroid carcinoma (FTC) only those homozygously deleted of the $TR\beta^{Pv}$ gene develop 100% of follicular thyroid tumor after 5 months, while heterozygous mice do not develop cancer.[7] Finally, these models do not always reproduce faithfully the specific pathways of development and progression of the human disease state such as angiogenesis and metastasis occurrence.

Using GEMM as a part of co-clinical trial remains currently challenging. First of all there is the necessity to establish cooperation between hospitals and research centers specializing in handling these mouse models. It is in fact necessary to have an adequate facility to engineer the mouse models, to analyze their genetic material, and it is also very important to establish a preclinical imaging laboratory to perform the *in vivo* analyses of tumor biomarkers, the radiochemistry of targeted drugs

with contrast agents of Magnetic Resonance, HFUS, radiotracers for PET or SPECT, and also to have available a dedicated mouse pathology facility. Furthermore, as suggested by Nardella *et al.* it is also very useful to be able to use comparative pathology centers (CPC) to support co-clinical trials in close consultation with the clinicians.[4]

Ideally, a closer collaboration between Physicians and Veterinary Doctors aiming at testing new drugs in the most efficient manner would accelerate the translation of new therapeutic discoveries to human medicine. An example of a co-clinical trial was the study on the mutation of KRAS in mice models of non-small cell lung cancer (NSCLC). This study was designed to determine if the MEK inhibitor selumetinib (AZD6244) could increase the efficacy of docetaxel, a standard-of-care chemotherapy for NSCLC. Pharmacodynamic studies, including positron-emission tomography (PET) and computed tomography (CT), identified biological markers in mice and patients that provided a rationale for the differential efficacy of these therapies in the different genotypes.[8]

In another phase II trial with NSLC harboring *EML4-ALK* fusion, the authors tested both in mice and patients the ALK inhibitor crizotinib against the standard-of-care cytotoxic agents docetaxel or pemetrexed. They showed that also in mice, crizotinib enhanced the overall survival in mice compared to the standard-of-care treatment.[9] The authors demonstrated the superiority of crizotinib to chemotherapy in ALK-rearranged non-small cell lung cancer and predicted strategies to overcome resistance.

PDX models used in co-clinical trial

PDX models also known as mouse "Avatar" have been recently used as a part of several co-clinical trials. PDX models could be very useful when patients are not in a state of good health to be enrolled in clinical trials or there is not an ongoing clinical trial for that patient. The concept is based on the fact that by implanting a patient tumor (biopsy or an excised tumor) in a mouse, it is possible to study a specific drug response and to test personalized therapy. It is possible in this way to predict either the efficacy or the lack of efficacy of the various pharmacological agents or other therapy strategies for an individual patient. PDX models are also useful to test the potential resistance of target pathways. It is also possible to generate from the tumor of a single patient different Avatar systems to simultaneously test several therapeutic approaches. Most cancers present a large number of mutations, and there is often more than one potential therapeutic approach for a single patient. PDX models are useful not only for the identification of drug targets but also to determine predictive biomarkers of disease or resistance. After first engraftment of a tumor in a mouse, the tumor is propagated through several generations (F0 to F3). Therapeutic agents are usually tested in F3; however, the use of further propagated tumor generations may be problematic. In fact during the passage through several generations, mouse stromal components may become dominant, so that the loss of the human tumor elements could create limitations in testing therapies. In PDX of non-small cell lung cancers, some authors tested the efficiency of three of the most commonly used drugs in first line chemotherapy. These authors developed a first generation NSCLC subrenal capsule xenografts, which where suitable for quick assessment (6-8 weeks) of the chemosensitivity of patients' cancers and selection of the most effective regimen in the clinics.[10]

Some other authors showed instead a combined approach of exome sequencing/bioinformatic analysis to verify the presence of somatic mutations and personalized xenografting to define therapy.[11] In these clinical studies, patients underwent conventional treatment while they tested the new therapy in a mouse Avatar, according to specific mutations found in the patients. Therapy was then made available to the patients selected for personalized therapy. Patients having colorectal, pancreatic cancer, glioblastoma, NSCLC, and melanoma were enrolled in this study, in which half of the patients received treatment according to results of the exome sequencing and the treatment on mice models. In most cases patient treatment was based exclusively on the Avatar mouse, where patients achieved a partial remission or a stable disease. Also this study had its limitations. In fact, patients' tumor xenografts do not always grow in immune compromised mice, and tumor engraftment efficacy may depend on the type of tumor. For some tumors, such as certain melanomas, lung and colorectal cancers, transplant efficiency can reach $\geq$75% with a time of tumor growth within few month. However, these rates depend on sample size and sample type

(*e.g.* pieces collected by surgery, biopsy, fine needle aspirate), tumor origin, and conservation of the patients' samples.[12] Therefore, the development of the Avatar model is expensive, and could require up to 6 months. Another limitation is related to the tumor microenvironment. Implanting a tumor subcutaneously does not reproduce the same microenvironment in which the explanted tumor was growing, and in xenografts models only few tumors metastasize. A good alternative to the subcutaneous xenografts model is to create an orthotopic model, which consists of implanting the tumor in the same organ of tumor origin, reproducing a similar tumor microenvironment to the original one.[13] Furthermore, this model often reproduces naturally the same metastatic process of the human disease.[14]

Another limit in the use of the PDX models is relative to the fact that these mice do not have an intact immune system; therefore, they are not useful to study the reaction of the immune system during anticancer treatments, which often could be helpful to better understand therapeutic mechanisms. PDX models are of limited use in screening immune mediate agents such as vaccines, immune modulators (anti-PD1) or agent activating the immune system like anti-CD40 antibodies. Today several strategies have been developed to modulate immune function in mice, for example one is to transplant purified human CD34+ hematopoietic stem cells into myeloblasted NSG/NOG recipients (BLT mouse strain developed by Jackson laboratories). Another way to reconstruct the immune system of a mouse and to use it for personalized medicine is to aspirate from the bone marrow of individual cancer patients hematopoietic stem cells and to inject them into the recipient mouse.[15]

Advanced therapy with ultrasound and microbubbles

The principle of gene therapy is to introduce genes or nucleic acids into cells to cure genetic defects. In particular, the success of gene therapy has been obtained with the use of viral vectors. In fact, viral vectors remain the best vehicles to introduce genes into cells for their ability to efficiently transfer genes with sustained and robust expression. However, systemic administration of viruses is thwarted by innate and adaptive antiviral immune responses. To circumvent this problem, it was demonstrated a unique intravenous site-specific viral delivery system for gene transfer both *in vivo* and *in vitro*, encapsulated in commercially available ultrasound contrast agents (microbubbles).[16-18] The Microbubbles (MBs) are able to entrap and to protect the viral vectors from the immune system.[19] The MBs are gas filled microsphere formed with a compatible shell composed of lipid, protein or polymers, capable of acting as ultrasound contrast agents. US contrast agents are delivered intravenously and can pass through the smallest of the vessels without obstacles because they typically range between 0.5 to 8 micron in size, and they contain high-molecular weight gases with less solubility and diffusivity, which improves MBs persistence allowing passage through the microcirculation (Figure 1).[16-20]

MBs have also been studied for their bursting behavior in response to destructive ultrasound for measurement of blood flow parameters and for drug and gene delivery. Ultrasounds help the delivery of genes in two ways. In the first place, the acoustic energy of US causes the transient opening of cell membranes, a process known as sonoporation. Sonoporation due to positive and negative pressure phases, produces pores in cell membranes facilitating the transport of genetic material through cell membranes, which are otherwise impenetrable.[21] In the second place, ultrasound causes the oscillation of MBs and rupture of the shell (cavita-

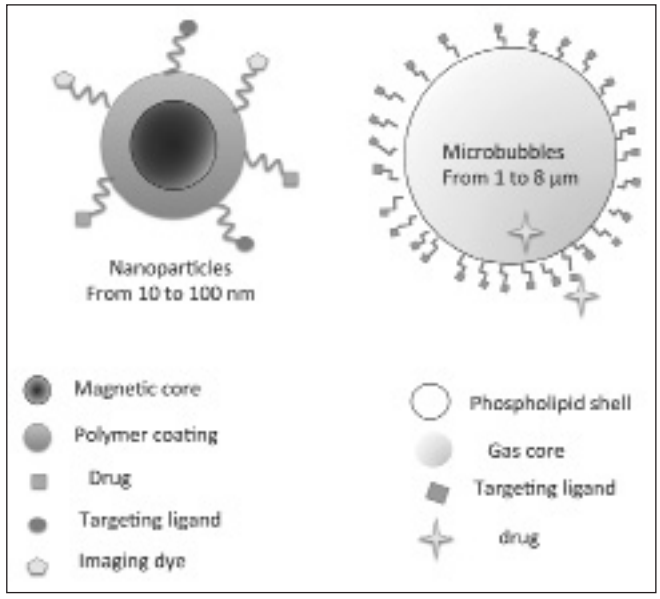

Figure 1.—Schematic diagram and comparison of nanoparticles' and microbubbles' composition.

tion), which causes the release of the genetic material from the MBs at the target site in a controlled manner.[21] In particular, the behavior of the microbubbles depends on the amplitude of ultrasound applied. At very low acoustic power (Mechanical Index <0.05-0.1), microbubbles oscillate in a relatively symmetrical and linear manner, and this produces ultrasound scattering equal to the transmitted frequency. At a slightly higher mechanical index of 0.1-0.3, the microbubbles become somewhat more resistant to compression than to expansion and they oscillate in a non-linear manner, backscattering a variety of frequencies. Higher acoustic pressures (Mechanical Index >0.3-0.6) destroy the microbubbles with diffusion of the gas via large shell defects or by complete fragmentation.

So, ultrasounds improve both the permeability of the target cells and the release of genetic material from the microbubbles.

The possibility of introducing microbubbles intravenously is a great advantage compared to using more invasive delivery procedures.

Several strategies have been developed to promote targeting of ultrasound contrast agents to specific regions of diseases'; however, all strategies may be grouped in two major approaches: passive targeting and active targeting.

Passive targeting exploits the chemical and electrostatic properties of the microbubbles' shell, resulting in the arrest of the MBs within the microcirculation. This method is based on up-regulation of receptors that bind non-specifically albumin or lipid components of the microbubble shell. Passive targeting does not exhibit molecular specificity, but allows a functional imaging of the pathophysiological condition that induces the over expression or up-regulated cell surface biomarkers.

Active targeting, on the other hand, is based on the attachment of specific antibodies or other ligands on the surface of the microbubbles. This leads to the accumulation of contrast agent targeted to specific sites due to the use of adhesion ligands able to recognize antigens peculiar to the disease. Potential ligands include antibodies, peptides, and polysaccharides.

With growing understanding of the molecular mechanisms underlying genetic diseases, gene therapy has been proposed as an effective approach. Currently, the main obstacle of the clinical application of gene therapy is not the lack of an ideal gene, but rather the lack of a safe and effective method to selectively deliver genes to target cells.

Ultrasound contrast agents have become important for their ability to directly provide the various classes of bioactive substances to a number of tissues, and they have become more and more popular for the targeted delivery of genes and for the administration of drugs. The system has been widely used in preclinical studies to enhance gene expression in a site-specific manner in a variety of organs. This technique offers advantages of high safety and allows to control spatially and temporally the effects of sonoporation in order to improve the localized tissue deposition of gene complexes (Figure 2). In fact, the MBs are destroyed only when they pass through the ultrasound beam for which the destruction site of MBs can be controlled by modulating the characteristics of the ultrasound field. In addition, direct deposition in the pathological site increases the effectiveness of the drug and reduces the effects associated to the systemic delivery.

An interesting application of gene delivery mediated by US is the eradication of human prostate tumor resistant to treatment.[17] Gene therapy is a promising strategy for the treatment of this tumor. The prostate gland is available by ultrasound and potential therapeutic genes can be directed to this organ using ultrasound after a simple intravenous injection. Prostate cancer (PC) is one of the most common cancers and is the second leading cause of death in men in the USA. Advanced PC is most commonly resistant to conventional anti-cancer treatments because it frequently overexpresses the anti-apoptotic protein Bcl-2 and or Bcl-x_L.

A secreted cytokine, that has broad-spectrum, cancer-selective and apoptosis-inducing properties that inhibits growth of PC cells, is the *mda-7/IL-24* gene. Intratumoral injection of *mda-7/IL-24* associated to a replication-incompetent adenovirus (Ad.*mda-7*) inhibited the growth of PC in immune-incompetent animals.

In contrast, *Ad.mda-7* showed to be ineffective in prostate cancer cells that overexpress Bcl-2 and/or Bcl-x_L, however intratumoral injection of a conditionally replication-competent adenovirus (CRCA) (a cancer terminator virus-CTV), expressing *mda-7/IL-24* caused growth arrest and apoptosis of the therapy resistant PC cells implanted in nude mice.

It has been shown that the MBs are the best candidates for the delivery of these genes. In fact, the complex MB/*Ad.mda-7* targeted in DU-145 cells using ultrasound has

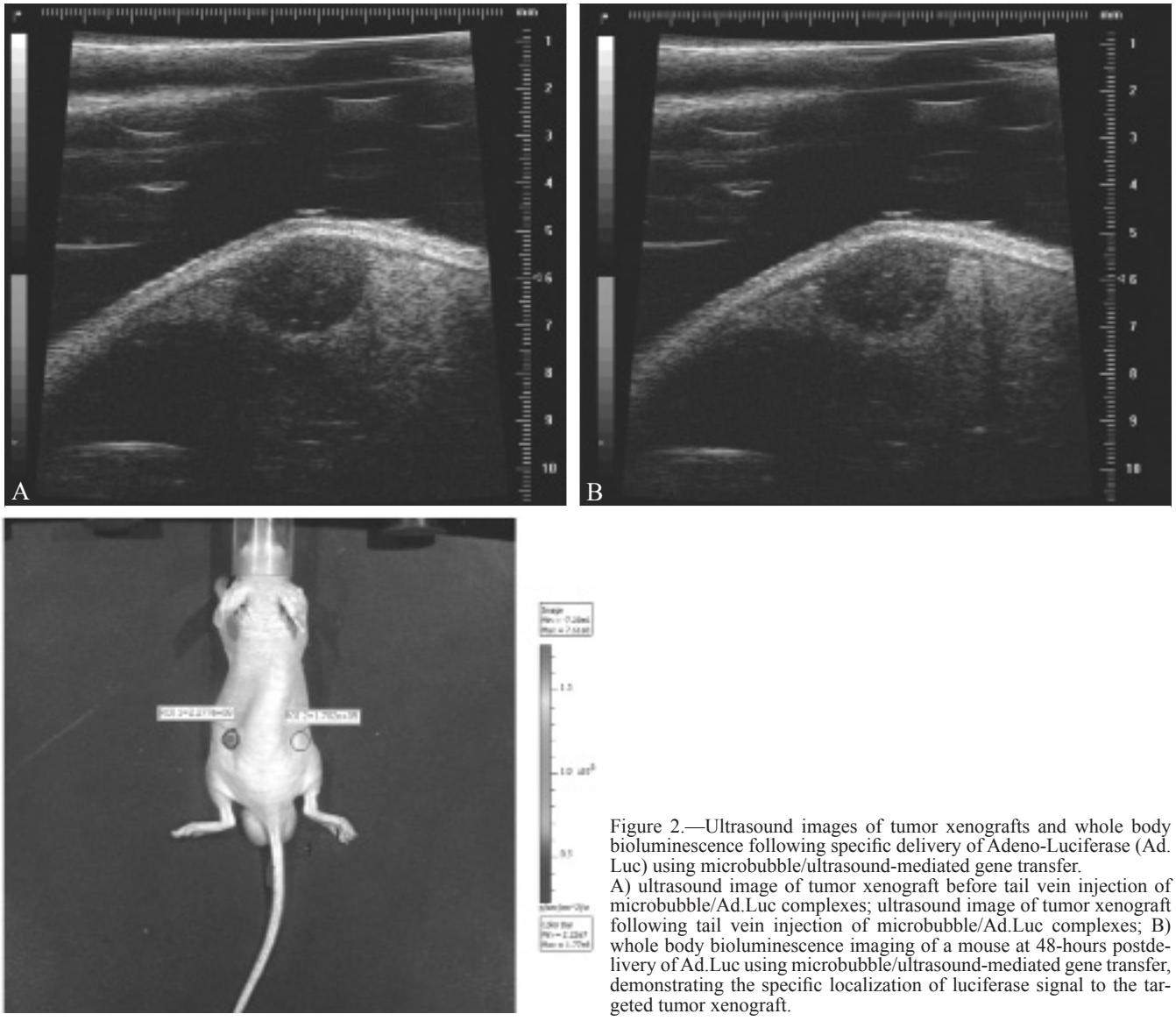

Figure 2.—Ultrasound images of tumor xenografts and whole body bioluminescence following specific delivery of Adeno-Luciferase (Ad. Luc) using microbubble/ultrasound-mediated gene transfer.
A) ultrasound image of tumor xenograft before tail vein injection of microbubble/Ad.Luc complexes; ultrasound image of tumor xenograft following tail vein injection of microbubble/Ad.Luc complexes; B) whole body bioluminescence imaging of a mouse at 48-hours postdelivery of Ad.Luc using microbubble/ultrasound-mediated gene transfer, demonstrating the specific localization of luciferase signal to the targeted tumor xenograft.

reduced the tumor implanted in nude mice. In addition, the delivery of MB/CTV guided by ultrasound has completely eradicated not only the cancer DU-145/*Bcl-*X_L resistant to therapy but also distant tumors that were not targeted by the ultrasound.

Therefore, the MBs by enclosing the adenoviruses in a gas-filled core allowed on one hand the protection of the virus from rapid degradation by the immune system and on the other hand to increase through ultrasound the delivery specificity of the Ads to the tumor.

A subsequent study has shown the further development of this effective treatment for prostate cancer resistant to therapy in combination with radiation therapy.[18, 21] These findings highlighted the therapeutic potential of this new image-guided gene transfer technology in combination with radiation therapy in patients with prostate cancer resistant to treatment.

Another study in 2012 investigated whether the outburst of the microbubbles using ultrasound could be used to improve the delivery of siRNA directed to the

EGF receptor (EGFR) in murine squamous cell carcinomas.[22] The inhibition of the Epidermal Growth Factor receptor (EGFR) is an established strategy for treating different types of cancers such as lung cancer, colorectal cancer and squamous cell carcinomas (SCC). Several pharmaceutical agents that can block the pathway of EGFR signaling like the monoclonal antibody cetuximab and the kinase inhibitor gefitinib have been developed. However, these molecules often are unable to block the pathway. For this reason, in 2012 other strategies such as RNA interference (gene silencing mechanism) have been explored to inhibit EGFR signaling. Direct injection of siRNA (short interference RNA) may seem appealing; however, RNAse may impair its action. For this reason microbubbles were used as a vehicle for EGFR siRNA. *In vitro* and *in vivo* studies were conducted to confirm down-regulation of EGFR by this delivery system. *In vitro* the microbubbles loaded with EGFR-siRNA and delivered to murine squamous carcinoma cells, reduced both EGFR expression and growth of EGF-dependent cells compared to control. *In vivo* the delivery of microbubbles, loaded with EGFR-siRNA and delivered to squamous cell carcinomas, decreased EGFR expression and tumor doubling time compared to control cells that instead received microbubbles loaded EGFR-siRNA, but without ultrasound or microbubbles loaded control siRNA and ultrasound. These results bear great promise for the future development of therapies based on direct gene delivery to tissues.

Nanocarriers in ultrasonic therapeutic systems

Drug resistance is a major obstacle for cancer curative chemotherapy. For this reason, in recent years, targeted therapy appeared to be a promising therapeutic choice for cancer treatment. The general approach is to develop vectors capable of locally releasing the drug/gene following the development of internal or external stimuli such as light, heat, and ultrasound. Imaging of the tumor should be performed before and during the release of the stimulus and also the biodistribution of the carrier is monitored through imaging to optimize the time of the release of the stimulus.[23] Microbubbles are commonly used as probes for intravascular ultrasound imaging and are becoming more popular tools for targeted drug delivery. However, due to their size microbubbles could remain confined to vessels and poorly reach tumor tissues; also their delivery is based on the EPR (enhanced permeability and retention) effects requiring hours or days for maximum accumulation. Since it is difficult to predict their *in vivo* absorption due to a number of factors such as the heterogeneity of the vascular bed, the tumor microenvironment, and the presence of infiltrating macrophages; the clinical importance of the EPS effect has been the subject of several recent scientific discussions.

For all these reasons nanocarriers of smaller size compared to microbubbles have been introduced as transport vehicles and delivery system for drugs (Figure 1). These nanocarriers in addition to target specific cells and tissues are able to preserve the biological activity of the drug during transportation. The nanocarriers have small dimensions and with long lifetimes of circulation, and display advantageous properties for diagnostic and therapeutic applications. They are able to cross the capillary walls and the walls of cell membranes to deliver the drug, thereby reducing side effects and increasing curative index. In addition these nanocarriers can be coated with specific ligands to adhere to the target tissue, thereby promoting intracellular uptake of the drug after insonation. Although the delivery system of the drug mediated by ultrasound is a very advantageous technique, there are several challenges still to be overcome. While the nanocarriers must be small in order to travel through the bloodstream, on the other side they must be large enough to prevent renal excretion, and stable to prevent the content from being released before application of the ultrasound pulse.[24]

Several nanocarriers were introduced and evaluated, including organic and inorganic materials. The family of nanocarriers includes *polymeric nanoparticles, nanoemulsions, liposomes* and *micelles*; recently also many inorganic materials are used as nanocarriers such as metal nanoparticles and nanocarriers based on carbon.

Polymeric nanoparticles

Polymeric nanoparticles include nanospheres and nanocapsules. The polymers mostly used for the delivery of drugs/genes consist of poly (lactic acid) (PLA), poly(e-caprolactone) (PLC) and poly(lactic-co-glycolic acid) (PLGA), these showed best properties of encapsulation and controlled release of the *in vitro* content.[25]

Compared with natural polymers, synthetic polymers have better purity and reproducibility. According to different needs, their structure can be changed to engineer nanoparticles that may avoid the immune system surveillance. For example copolymerized polymeric nanoparticles with polyethylene glycol (PEG) can avoid recognition by the phagocytic mononuclear cells. The polymeric shell may also improve the stability of nanocarriers and increase their ability to resist the ultrasound pressure field.

Recent research has developed preparations of hybrid compounds with metals and/or active principles, allowing the use of nanoparticles as theranostic agents. The metals commonly used include gold, iron, silver, and gadolinium. Such theranostic agents can be used both to diagnose and monitor treatment response of cancer using magnetic resonance imaging (MRI) and to deliver encapsulated therapeutic agents.

Perfluorocarbon nanoemulsion

The family of liquid perfluorocarbons (PFCs) includes Perfluorodecalin (PFD), Perfluorooctyl bromide (Pfob), Perfluorohexane (PFH), Perfluoropentane (PFP), Perfluorotributylamine (PFTBA) and Perfluoro-15-crown-5-ether (PFCE). The PFCs are fluorinated compounds initially used in clinics as gas/oxygen carriers and today are considered as contrast agents for echography, magnetic resonance imaging and targeted therapy. A PFC nanoemulsion is prepared starting from a mixture of hexane and pentane perfluorinated, the nanoemulsion is formed because of the self-assembly properties of the polymer and when the solvent is replaced. The PFC particles can infiltrate the arterial walls, cross the internal elastic lamina, and bind and localize molecular epitopes in intramural tissues. Similarly, PFC nanoparticles targeted for angiogenesis markers have been used successfully to detect neovascularization around tumors implanted in athymic mice using ultrasonic scanners.[26]

Liposomal nanocarriers

The liposomes (vesicles in double lipid layer) are colloidal structures consisting of a mixture of phospholipids and cholesterol in aqueous solution.[27]

The major component of the lipid bilayer is phosphatidylcholine, a natural phospholipid that contains a phosphate group linked to a hydrophobic section. Liposomes are commonly prepared using the method called "film hydration": the various components are combined codissolving the lipid in an organic solvent. Subsequently, the organic solvent is removed from the vacuum deposition of the films. When all the solvent is removed, the mixture of solid lipid is hydrated using an aqueous buffer. Lipids immediately swell to form liposomes. Liposomes are effective carriers of drugs; their multifunctional characteristics can be obtained by modifying the lipid composition of the shell (double layer). Liposomes have low immunogenicity, good biocompatibility and degradability, and are often used as a shell for nanobubbles. Compared to materials coated with polymers (polymer-coated materials), the liposomal nanocarriers improve the intensity of the imaging signal. In a study by Piao *et al.* human serum albumin-coated nanoparticles with lipid-HSA LNPs were prepared to contain siRNA (HSA-LNPs-siRNA). The nanoparticles were loaded with phrGFP-targeted siRNA. The study showed a significantly reduced expression of phrGFP mRNA by HSA-LNPs-siRNA in phr-GFP MCF7, MDA-MB-231 and SK-BR-3 transfected cells with respect to control. In a phrGFP-transfected MCF-7 tumor xenograft model, tumor fluorescence was decreased after IV administration of HSA-LNPs-siRNA at a dose of 3 mg/kg compared to siRNA alone. HSA-LNPs-siRNA showed a superior pharmacokinetic profile compared to control siRNA at a dose of 1 mg/kg. These results demonstrated that HSA-LNPs could be considered a new non-viral carrier that can be used for delivery of siRNA in tumor cells of the breast.[28]

Micelles

A micelle consists of a series of amphiphilic surfactant molecules aggregated spontaneously in water, forming a spherical vesicle. The "core" of the micelle is hydrophobic and it may contain hydrophobic drugs. A micelle is defined by its molecular size and other characteristics of the surfactants. Polymeric micelles formed from poly (ethylene oxide) -b-poly (propylene oxide), poly (ethylene oxide) -b-poly (ester) and poly (ethylene oxide) -b-poly (amino acids) have great promise for the delivery of drugs.[29] Several studies have demonstrated the efficacy of ultrasound-mediated delivery of drugs/

genes using micelles as carriers for the treatment of tumors *in vivo*. Diaz de la Rosa *et al.* have prepared nanomicelle of loaded drug with a diameter of about 10 nm.[30] Husseini *et al.* have prepared nanomicelle loaded of drugs by co-incubating nanomicelles and anticancer drugs, reducing the side effects of chemotherapy.[24] It has also been shown that copolymers micelles as PEO-PPO (PPO-PEO-block copolymers) are more secure, and kinetically stable with better capacity of solubilization than regular micelles.[31, 32] Despite these advantages, PEO-PPO-block copolymers still suffer from limitations such as low stability and short residence time; all factors that limit their application.

Albumin nanoparticles

Albumin is an excellent carrier that can be used for drug delivery. It is promising for being non-toxic, non-immunogenic, highly biocompatible and easily biodegradable. Albumin nanoparticles have been measured with dynamic light scattering method to have a 100 nm diameter. They have gained more and more importance for their high release drug capacity and to elicit almost no side effects. Michaelis *et al.* have developed nanoparticles of human serum albumin (HSA-NP) using nanoparticles of Apolipoprotein-E, which could cross the blood-brain barrier with loperamide (which exerts an antinociceptive effect).[33] Apo-E has been shown to mediate transfer of drugs across the blood-brain barrier. Apolipoprotein-E has been associated with HSA-NP nanoparticles loaded with loperamide by means of a chemical method. Using the HAS-NP/loperamide nanoparticles, immediately after their intravenous injection, it was observed an antinociceptive effect in ICR mice; demonstrating that the nanoparticles have crossed the blood brain barrier.[33]

A different approach to the traditional drug delivery is the use of ultrasound to implode the microbubbles in nanoparticles.[34] Conversion from micro to nano allows both carriers to bypass the EPR effect, using an external trigger, and both to penetrate more easily the tissue barriers due to their small size. In this way, nanoparticles, filled with drugs, first penetrate the diseased tissue and then release the drug when the core is converted into a gas.

Huynh *et al.* at the University of Toronto have created multimodal microbubbles incorporated in a shell of porphyrin-lipid around a perfluoropropane gas. The gas provides contrast ultrasound images while the porphyrin gives the shell fluorescent and photoacoustic properties.[34]

When these multimodal microbubbles are subjected to low-frequency ultrasound, the porphyrin microbubbles (pMB) implode to nanoparticles (pNP) with a diameter between 5-500 nm. The resulting nanoparticles retain the photoacoustic and fluorescent properties; these properties make it possible to demonstrate the real conversion of the pMB to pNP.

In particular, the pMB are exposed to 1 MHz, high-duty-cycle (50%), ultrasonic (2 W cm-2), named as "conversion US" in 2 sec pulses and characterized after sonication with zero, one, three or ten pulses. After ten pulses, most of the pMB are converted into pNP. This conversion is possible in tumor-bearing mice using photoacoustic imaging. In fact, the resulting pNP penetrate preferentially in tumor tissues highlighting them with optical and photoacoustic imaging.

The specific mechanism that drives the conversion of the multimodal microbubbles from micro to nano, has yet to be studied, in addition the conversion is specific to pMB. Conventional microbubbles formed by a shell of phospholipids without the attached porphyrin group, after sonication with "US conversion", form nanostructures similar to the pNP, but this nanostructures do not display any therapeutic function due to the absence of the porphyrin component.

The pNP based on porphyrin have proven interesting for imaging and treatment of diseases, especially for cancer, due to their multifunctional nature. The presence of the porphyrin has transformed a conventional microbubble from the unimodal ultrasound contrast agent and delivery vehicle, without any function after the ultrasound mediated outburst, to a trimodal contrast agent with both imaging and therapeutic properties (theranostic). These considerations introduce a new theranostic strategy in a research and health care field that is constantly evolving such as cancer management.

A further refinement of this technique will be to produce nanoparticles in which the high therapeutic and diagnostic payloads within the pM will generate smaller sized nanoparticles to increase the delivery efficiency of the image-guided drug.

Innovative design of microcarriers for drug or gene delivery to overcome biological barriers.

The advantages shown by micro and nanocarriers for drug delivery have opened new avenues for site-specific delivery of bioactive drugs.[35] The microcarriers are considered safe and tolerable vehicles, but despite these features, these carriers face a number of sequential biological barriers that limit their site-specific bioavailability and thus adequate therapeutic results. These obstacles include opsonization and subsequent sequestration by the mononuclear phagocyte system (MPS), non-specific distribution, blood vessels flow limitations, cellular internalization, and drug efflux pumps.

It is necessary, for an innovative design to take into account all the biological barriers that microcarriers may meet following intravenous administration.

Innovative designs, such as the use of non-traditional geometries to improve vascular dynamics or functionalization with biomimetic membranes to prevent the uptake of phagocytes, have shown several advantages with respect to conventional carriers [35].

First, the microcarriers are subject to opsonization and sequestration by MPS, resulting in increased accumulation in organs such as the spleen and liver, and contributing to a non-specific distribution in healthy organs. Several strategies have been used to "camouflage" the microcarriers such as PEGylation, which provides a hydrated surface that hinders the formation of a protein corona prolonging the circulation lifetime of microcarrier/drug complexes from minutes to hours,[36] or conjugation of the self CD47 peptide on their surface so that macrophages identify them as self and avoid phagocytosis.[37] Finally, coating with cell membranes extracted from autologous leukocytes or red blood cells that provide instead a biomimetic surface, thereby avoiding uptake from macrophages.[38]

Because the dynamic flow in blood vessels, and adhesion properties, microcarriers are all heavily dependent on their geometry and size.[39] Several scientists studied a non-spherical design of the microcarriers for drug delivery. In fact, numerous discoveries have shown that non-spherical particles under a flow show dynamic rolling and tumbling, compared to spherical ones that flow at a certain distance parallel and away from the vessel wall.[40]

Even cellular internalization proves to be a formidable barrier. Indeed endocytosis of microcarriers leads to their invagination in the membrane and the formation of intracellular vesicles, which fuse with lysosomes. The high acidity of the lysosome environment, also rich in enzymes, causes damage to the microbubbles' payload especially if it consists of genetic material. A strategy to prevent the degradation of the payload is the incorporation of membrane peptides destabilizing like INF7, H5WY and GALA inducing endosomal escape [41] or the incorporation of cationic peptides such as poly-ethylene imine (PEI) that instead guide the therapeutic agent release from the endosomal compartment.

The last obstacle is represented by the drug efflux pumps that confer multidrug resistance (MDR) because they excrete the drug upon entry into the cell. To circumvent this, microcarriers coupled with inhibitors of drug efflux pumps have been developed which are more cytotoxic making them more effective than control microcarriers.[42]

However, in spite of all the improvements in the design of these microcarriers, if all of the biological barriers that a drug encounters during its delivery are not considered when designing a microcarrier, drug delivery systems will continue to fail in their clinical application. Only a better understanding of the biological processes that regulate these barriers will allow both the development of more appropriate carriers and better site-specific delivery.

Ultrasound at low-intensity for cancer therapy

In recent years, the preclinical field established four new techniques for cancer therapy, such as sonodynamic therapy, ultrasound mediated chemotherapy, ultrasound-mediated gene delivery and antivascular ultrasound therapy.[43] Unlike the techniques previously described, these techniques utilize low-intensity ultrasound.[44] In each therapeutic modality, theranostic contrast agents composed of microbubbles pose both a therapeutic role and a diagnostic/imaging role. The development of these agents is important because it establishes a therapeutic-diagnostic platform that is useful to monitor the success of anti-cancer therapies.

There is no clear definition of low-intensity ultrasound, but generally it corresponds to an intensity less than 5.0 W cm-2, equivalent to a root-mean-square pressure amplitude of about 0.3 MPa.

The insonation of tumors with low-intensity ultrasound is easy to perform, because it does not require a focused beam, the apparatus is not expensive, and the effects on adjacent normal cells are minimal. The dif-

ference with high-intensity ultrasound is that times of treatment are longer.

The four aforementioned techniques produce bioeffects that induce the death of cancer cells, and we will focus our attention on sonodynamic therapy and antivascular ultrasound therapy.

Sonodynamic therapy

*S*onodynamic therapy (SDT) is derived from photodynamic therapy. While in photodynamic therapy, photosensitizers are excited by light to produce reactive oxygen species, SDT uses ultrasound to produce cavitation and to induce sonosensitizers to produce free radicals that kill cancer cells rapidly growing.

An important development of SDT is the combination of sonosensitizers with microbubbles contrast agent. The combination of sonosensitizers with microbubbles contrast agent creates a theranostic agent; because through the microbubbles, loaded into the tumor vasculature, the agent can be monitored using ultrasound imaging, and once it is detected in the site of interest sonodynamic therapy can be initiated. In addition to cytotoxic effects, other possible effects on tumor growth have also been evaluated, such as thermal effect and antivascular ultrasound.

In recent years, considerable data have been published using different sonosensitizers applied to different types of tumors, although several studies must still be performed to assess the effectiveness of the different sonosensitizers that have been developed so far.

To date, studies of SDT have been performed only in small laboratory animals implanted with subcutaneous tumors; future studies should be performed in larger mammals before it will be tested in phase-I human clinical studies.

Antivascular ultrasound therapy

Antivascular ultrasound therapy also uses microbubbles as theranostic agents.

It has been hypothesized that a solid tumor should be constituted by at least two cellular compartments; one containing the neoplastic cells, and the other containing the endothelial cells from tumor neovascularization. Unlike normal tissues, tumor tissues have a fragile vascularization and abnormal branching patterns. Tumor neovascularization can be detected by ultrasound using microbubbles to differentiate vascular from avascular regions within the tumor.[45] After intravascular injection, the vascularized regions are accessed using a B-mode image of routine and power Doppler. In this case, the combination of ultrasound and microbubbles was used to damage tumor vasculature causing cell necrosis and subsequent reduction of the tumor mass. In several studies of murine melanoma models, it has been shown that the use of ultrasound on the tumor in the presence of microbubbles has a significant antivascular effect.[46-50] The predominant effect of insonation, in the different studies, was the irreparable dilatation of tumor capillaries with intracellular edema. In particular, every minute of insonation decreased perfusion of the tumor by about 25%, which was followed by necrosis of the neoplastic cells. Interestingly, in these theranostic studies, the vascularization in adjacent normal tissues was not affected by the ultrasound treatment. In these studies it was noted that the action of ultrasound antivascular low-intensity was increased when the insonation occurred at 3 MHz than that at 1 MHz and the temperature of the tumor increased by 5 °C·min^{-1} and 2 °C·min^{-1}.[48] The main biological effect recorded on endothelial cells is the thermal effect. In fact, blood flow in tumor vessels is slower than normal vessels causing further interactions between ultrasound and microbubbles resulting in damage caused increased temperature to the endothelial cell coatings. The damage is less in normal capillaries, where circulation is faster and any increase in temperature is smaller and has no biological effect.

Besides the thermal effect also cavitation of the microbubbles is a biophysical interaction mechanism. Studies using continuous waves of ultrasound emphasize the creation of thermal effects while pulsed ultrasound determines cavitation. While it is difficult to separate the two effects, both play a role. Low-intensity ultrasound with continuous waves cause the oscillation of microbubbles with long intervals leading to significant heating effects. High-intensity ultrasound with shorter pulses instead yields the collapse of the microbubbles causing an inertial cavitation.

Antivascular ultrasound therapy offers some advantages but it also has several limitations. The advantage is that the effect is generic and not specific to any type of cancer. Antivascular ultrasound requires access to the endothelial cell surface unlike drugs that should instead

penetrate. A limitation of antivascular ultrasound is that susceptible tumor neovasculature has to be present for the therapy to be effective.

Antivascular ultrasound therapy is very powerful but it remains to clarify the mechanism of interaction of different intensities of ultrasound. Additionally, it should be optimized so that the disrupting effects on the vasculature are maximized while minimizing damage to adjacent normal tissues.

MRI nanoparticles for theranostic applications

Drugs available for cancer treatment are not completely effective; hence researchers have been prompted to look for new therapeutic strategies. The main limitations of drugs currently used in clinical oncology are: 1) non-specific molecular targeting of cancer cells; and 2) systemic toxicity.[51] In recent years, in an attempt to overcome such limitations, a new frontier for pharmacological studies has been identified in the preclinical imaging of animal models of human oncologic diseases. Moreover, the gap between diagnosis and the beginning of treatment is another factor that reduces therapeutic efficacy. Theranostics describes any material or substance able to exert a therapeutic effect in the target tissue while simultaneously diagnosing the pathology by imaging. Nanotheranostics exploits nanotechnology for treating and monitoring disease in real-time.[52] Nanoparticles (NPs) are solid colloidal particles ranging in size from 1 to 1,000 nm, and are usually classified on their size, shape, charge and surface chemistry.[53, 54] In theranostics, the NPs can be "organic" (*e.g.* lipids, proteins, polymers) or "inorganic" (magnetic and non-magnetic NPs).[53, 54] Among the potentials of NPs are: the possibility to include within the core or in the outer structure contrast agents and/or a therapeutic cargo, to functionalize them with targeting elements, and, for some nanomaterials, to be used for thermal therapy.[54]

Magnetic resonance imaging (MRI) is a powerful tool used in diagnostic imaging, and it offers the advantages of a high spatial resolution, the absence of ionizing radiation, and the production of tomographic images.[55] Theranostic applications of MRI for cancer therapy are based on magnetic NPs (MNPs), approximately of 10 to 100 nm, which display the double function of imaging contrast agent and anticancer therapy.[56, 57] In fact, they are able to induce cell death by heating, as well as to release drugs, when subjected to a high frequency magnetic field.[56, 58] The MNPs show enhancing properties in MRI scans, in the tissues where they accumulate, due to the presence of magnetic elements either in their shell or in the core, which causes the shortening of T1 or T2 relaxation times.[55, 59] The MNPs accumulate in target tissues through: 1) passive targeting; 2) active targeting; and 3) targeting due to an external magnetic field.[53] Passive targeting takes advantage of the lack of architecture of blood vessels that characterize most solid tumors. This phenomenon is called "enhanced permeability and retention" (EPR), and, because of this effect, molecules larger than 40 kDa accumulate in the tumor, while avoiding healthy tissues.[60] Active targeting is based on molecules bound on the NPs surface, which recognize tumor-selective receptors, in order to exploit their anticancer proprieties in the target tissue and to prevent systemic side effects.[53, 61] Targeting with an external magnetic field combines active targeting with the magnetic field ability of concentrating the NPs in the tumor lesion.[53]

In the following paragraphs, the state of the art about MNPs theranostic applications for cancer therapy will be discussed.

MRI nanoparticles and anticancer drugs

Chemotherapy is one of the most commonly used approaches against cancer. The main issues of chemotherapy in cancer therapy are the systemic effects that it produces, *i.e.* damage to healthy tissues and/or systemic side effects. In order to construct a specific drug delivery system able to bypass body barriers, and to elude drug degradation, magnetic NPs capable of incorporating several chemotherapies such as Paclitaxel, Doxorubicin (DOX), and Gemcitabine (GEM) have been developed.[55]

These nanotechnology-based drug delivery systems have minimized the distribution of chemotherapies in normal tissues and allowed their delivery beyond the body barriers in the target tissues.[60] For example, in brain tumors the presence of the blood-brain barrier (BBB) as well as the expression of drug efflux pumps doesn't allow the passage of many anti-cancer molecules.[62] For example, poly – (methacrylic acid) – polysorbate – 80-grafted-starch has been developed as a nanotheranostic system that allows DOX delivery across BBB

for brain metastases therapy, since free DOX is not able to penetrate the BBB, and this system is also able to co-incapsulate Gd-DTPA as a MRI contrast agent.[62]

Magnetic iron oxide nanoparticles (IONPs) combines imaging contrast properties with drug delivery capabilities, overcoming the tumors' intrinsic barriers.[57] The complex of IONPs and gemcitabine (GEM), combined with the natural ligand of uPAR expressed in pancreatic cancer, has been demonstrated to inhibit tumor growth in a xenograft mouse model of pancreatic cancer.[57] The properties of Super Paramagnetic Iron Oxide (SPIO) NPs as surface charge, magnetic assets and size, make SPIO optimal not only for cancer detection, but also for the release of drugs and gene therapy.[55] When SPIO NPs are administrated *in vivo* by intra-vascular injection, they accumulate in the tumor mass by the EPR effect, and they are able to bypass drug resistance mechanisms such as P-glycoprotein efflux pump.[63] The SPIO can be loaded with different types of chemotherapies for the treatment of oncological diseases *in vivo*.[64] Drug biodistribution can be studied combining therapeutic agents with SPIO in a polymeric vehicle.[52]

In order to stabilize NPs under physiological conditions, and to optimize their function, it is necessary to functionalize them with polymers, such as polyethylene glycol (PEG) or poly–(lactic-co-glycolic acid) (PLGA).[65] The PEG, for example, is one of the most commonly used polymers, and it has important functions such as improving drug encapsulation and subsequent release, and extending the circulation half-life of the NPs by preventing the opsonization process and avoiding the uptake by the reticuloendothelial system *in vivo*.[56, 66] The HER2-targeted PLGA−PEG block copolymer NPs, encapsulating $MnFe_2O_4$ and DOX, exhibit ultrasensitive detection by MRI, and excellent tumor growth retardation both *in vitro* and *in vivo*, with high specificity for breast cancer.[52, 67] The SPIO combined with PLGA, and loaded with the anticancer Paclitaxel, is able to delay tumor growth in mouse xenograft models of colon carcinoma.[68] The PEGylated $FePt@Fe_2O_3$ NPs, thanks to their hydrophobic oleic acid layer, are able to incorporate DOX. The specific release of DOX occurs with a pH-dependent mechanism, and it is favored by an acid pH, typical of tumor environment.[65] PEGylated NPs composed by GEM-50-monophosphate and Gadolinium showed a higher anticancer efficacy in breast cancer xenograft mouse model compared to free drugs, as well as a better enhancement than clinical contrast agents.[65] Furthermore, the PEGylated surface of NPs offers greater retention time within the tumor.[65]

MRI nanoparticles for photothermal therapy

Photothermal therapy (PTT) is an anti-cancer treatment that uses heat production through different sources, such as: radiofrequency, high-intensity focused ultrasonography, microwave, alternating magnetic field, and laser.[69] The PTT must be supported by valid methods to identify the tumor location, to monitor therapy in real time, and to evaluate the effectiveness of treatment after therapy.[70] PTT uses near infrared (NIR) laser irradiation to heat NPs and selectively kill the cells that incorporated NPs. Several strategies have been developed to bridge together cancer PTT and multimodal imaging.[56]

MoS_2 is a graphen-like nanomaterial used for PTT due to its high NIR absorption ability.[71] A dual MRI/Photoacoustic tomography (PAT) system has been used to realize effective magnetic targeted photothermal ablation of cancer while performing imaging diagnosis *in vivo*, with a complex of MoS_2 and Fe_3O_4 NPs.[71] Combining ultrasonography, laser irradiation and MRI, gold nanoshelled NPs have been used to obtain effective thermal ablation in tumor bearing mice with a precise focus on tumor location.[70]

Among the compounds that have displayed the best outcome in the context of PTT, is WS_2-IO@MS-PEG, in which WS_2 nanosheets, pre-adsorbed on their surface with IONPs, are coated with a mesoporous silica shell, on to which PEG is attached. This system has been tested in tumor bearing mice, and drug delivery and thermal ablation efficacy has been evaluated, demonstrating no toxic effects.[67]

Dual metal-organic-frameworks (MOFs) NPs, with Prussian blue core, represent one of the most innovative systems that combine imaging with drug delivery performance. This compound can be used both as MRI imaging contrast and as fluorescence optical imaging contrast. Furthermore, it can be loaded with anticancer drugs, performing both chemotherapy and PTT.[72] The effectiveness of combined drug delivery and PTT has been also assessed using carbon nanomaterials, such as graphene oxide (GO). Due to its optical absorption propriety, the GO can combine both photodynamic and

photothermal hyperthermia, leading to an optimal therapeutic efficiency.[56] The PEG-modified GO@Gd combined with DOX showed ability to detect tumor location on MRI, and high photothermal-chemotherapeutic efficacy with low toxicity, in a xenograft mouse model of sarcoma.[59]

Magnetic hyperthermia treatment employs an alternating magnetic field to raise the temperature of the NPs. Since cancer cells are more sensitive to heat compared to normal cells, this treatment appears to be safe and in some way specific for tumor tissues, even if specific magnetic frequencies should be selected to avoid healthy tissue damage. However, to ensure a successful outcome, *i.e.* a temperature rise able to produce cell death, a sufficient amount of MNPs should be accumulated into the tumor.[73]

Recently, a liquid phase system composed by Fe_3O_4 NPs, calcium phosphate cement and PEG-600 has been developed, which can be injected directly into the tumor mass *in vivo*. This material, once inside the tumor mass, turns into solid phase, thus avoiding the spread to the surrounding tissues. By applying an alternating magnetic field, this complex generates heat causing the death of cancerous cells.[74] Another advantage of magnetic hyperthermia is the increasing drug penetration due to destruction of the extracellular matrix.[64] The DOX-loaded multifunctional liquid to solid phase transitional magnetic material can generate heat when exposed to alternating magnetic field. This method improved both thermal ablation and the DOX delivery. In fact, DOX release percentage and rate was higher in tumor xenograft mice exposed to alternating magnetic field than in non-exposed mice.[75]

In spite of PTT and magnetic hyperthermia being among the most recently tumor ablation methods, each of them is characterized by its own main disadvantages. For PTT, high doses of NIR laser irradiation can damage surrounding tissues, whereas for magnetic hyperthermia, high concentrations of NPs are required, which may produce systemic side effects. These drawbacks might be overcome by dual modality treatment using IONPs, which show both magnetic and thermal features, as demonstrated in tumor bearing mice. Espinosa *et al.* in 2016 through an alternating magnetic field and NIR laser irradiation at a wavelength of 808 nm, demonstrated that it is possible to amplify hyperthermia 2 to 5 fold compared with magnetic stimulation alone, with complete tumor eradication *in vivo*.[76]

MRI gene therapy

The aim of gene therapy is the replacement, the silencing or the regulation of a pre-existing defective gene through the administration of genetic material. Nucleic acid internalization is subjected to degradation by immune system, thus reducing gene therapy efficacy.[53, 77] Both viral and non-viral vectors have been developed to promote genetic materials delivery *in vivo*. Viral vectors, even if effective in gene delivery, often cause an immune response.[77] To overcome this issue, MNPs can be functionalized to obtain their accumulation in tumors and to achieve gene delivery by magnetic field application.[52, 53] In order to produce a successful effect, it is necessary that such loaded-NPs can penetrate into the cell nucleus, before releasing the genetic material. This goal is obtained through conjugation of peptides.[64] The concentration of MNPs in tumor mass and gene transfer by means of a magnetic field is called "magnetofection", which has the advantage of realizing a specific-site therapy, and it is not applicable to "diffuse" tumors.[78]

Small interfering RNAs (siRNAs), has been recently one of the main protagonists in the scenario of gene therapy. siRNAs are able to silence pathways that are up-regulated in a cancer cell, but when siRNAs are injected intravenously, they are rapidly degraded.[79] For this reason, and to improve the transduction efficacy of siRNAs, different systems have been developed. Polyethyleneimine (PEI) possesses large amounts of functionalized amine groups that allow building up complex molecules with nucleic acids that can be used to deliver siRNA.[79]

Tripeptide arginine glycine aspartic acid (RGD)-PEG-g-PEI-SPIO exhibited high efficacy as MRI contrast agent as well as inhibitor of tumor growth in a mouse model of hepatocellular carcinoma, suppressing *survivin,* an anti-apoptotic protein overexpressed in this histologic type.[79] Nevertheless, PEI combined with NPs showed high toxicity. A disulfide-based polycation (SS-PEI), incorporated in SPIO, represented a useful strategy for gene delivery and MRI imaging with reduced toxicity, in tumor bearing mice.[80]

Tumor suppression through gene therapy can be carried out through the down-regulation of angiogenic factors, which promote tumor aggressiveness and metastasis capability. The PEGylated magnetic mesoporous silica NPs PEI-capped, conjugated with fusogenic

peptide (KALA)-functionalized siRNA delivery system, showed a high anti-tumor efficacy through VEGF down-regulation in a mouse model of ovarian cancer.[81] Finally, chitosan NPs have been developed to obtain optimal results in gene therapy, due to the chitosan molecular weight and to NPs degree of deacetylation; however, they should be tested in further *in vivo* studies [82] to determine their efficacy and safety.

Toxicity of MRI nanoparticles for theranostic applications

The principal aim of nanotheranostics is to improve and to anticipate treatment, and response, while minimizing systemic toxicity and side effects. Gadolinium, which is commonly used as contrast agent in clinical imaging, may be toxic in some patients, particularly with some insufficiency and it is also harmful to the environment.[56] On the other hand, the toxicity of NPs is highly dependent on structural properties, dosage, solubility, surface chemistry, administration route, biodegradability, pharmacokinetics, and biodistribution.[83] The cytotoxic effect of MNPs is mainly due to the production of reactive species of oxygen that induce cell death, when the dosage exceeds 100μg/mL.[83] However, reactive species of oxygen may be desired to kill cancer cells.[84] MNPs do not generate particular toxic effects *in vivo*, as showed by the absence of major changes in mice bodyweight.[66, 67, 70, 72, 81]

In order to reduce as much as possible adverse effects of NPs, their production was directed towards components already existing in nature, such as IONPs.[56] However, *in vivo* studies about the toxicity mechanism of IONPs are still conflicting.[85] The IONPs administered in animals showed different toxicity degrees depending on NPs size, crystalline phase and dissolution rate, administered dose, and age or preexistent pathological state of the animals analyzed.[85]

Studies on SPIO showed that, in addition to the physical and chemical composition and dose of NPs, different cell types show different degree of cytotoxicity. Indeed, cardiac cells do not suffer any toxic effect from SPIO, whereas SPIO produced severe disruption of the actin cytoskeleton in renal and cerebral cells *in vivo*.[86] Additionally, PEI and PEG conjugations have demonstrated reduced *in vivo* toxicity.[66, 78]

In order to generate magnetic hyperthermia a definite amount of NPs in the tumor are required, which is limited by the maximum tolerated dose, and an increased magnetic field, which may produce a damaging heat level in the surrounding tissues.[66] In a similar way, also magnetofection is limited by the maximum tolerated dose. Taken together, these results encourage the implementation of methods for theranostics, in order to minimize the systemic and toxic effects induced by conventional chemotherapies.

A new drug delivery method: MRI-guided focused ultra sound

A major goal of pharmacology is to achieve adequate concentration of the drug in the site of disease by limiting toxicity to normal tissues.

It has long been understood that the local drug delivery with real-time imaging is a promising strategy in achieving this goal.[87]

Ultrasound and Magnetic Resonance Imaging (MRI) play an important role in this field.[88] Ultrasound, as we have demonstrated, when used in conjunction with microbubbles is a very useful method for drug delivery, allowing the release of biological drugs from the vehicle at the site of interest thereby increasing local permeability of the cell membrane.[16-18, 21] However, after the outburst of the microbubbles and the release of the drug the hyper-echogenicity disappears. Therefore, ultrasound imaging can only be used to follow and image the microbubbles at the target disease site, but distribution and further monitorization of the delivered drug can not be determined.

It is possible instead by using MRI to not only deliver the drug, but also to monitor and follow biodistribution of the delivered drug. MRI offers several advantages, for example, it is completely non-invasive, it has a high spatial resolution, a high variety of biomarkers, and a temporal resolution sufficient to measure changes in the distribution of the drug.

The best way to monitor the pharmacodynamics and pharmacokinetics of a drug is to follow the same drug using contrast agents conjugated with the drug itself. A new approach includes loading the drug with the contrast agent in the same delivery system. For example, loading of $MnSO_4$/doxorubicin in liposomes to monitor *in vivo* the concentration and the release of the drug has been recently explored.[89]

Another MRI application is the focal drug delivery through MRI-guided focused ultrasound.[90] The term focal drug delivery has been introduced to describe focal targeting of drugs in tissues with the aid of imaging and focused ultrasound. The focused ultrasound (FUS) or therapeutic high intensity focused ultrasound (HIFU) is a non-invasive medical treatment that allows focusing the ultrasound at a given point of the body. The energy levels transported by the ultrasonic beam are of different orders of magnitude compared to those of diagnostic standards. These ultrasound beams may be delivered to a small volume sparing surrounding tissue. The main effects of the FUS beam are heat, mechanical effects, cavitation, and chemical effects. Heat is the most common physical effect generated by sound waves into the body. When the heat generation rate is higher than the dissipation rate of the body, the body temperature increases significantly; temperatures higher than 43 °C, if sustained for a prolonged period can be harmful. The mechanical effects, such as breaking of bonds, may occur when the amplitude wave ultrasound is significantly high. Cavitation occurs when the ultrasound beam of sufficient intensity passes through a liquid in which gas bubbles have been generated; the alternation of high and low pressure periods force the bubbles to contract and expand. During contraction, the internal pressure of the bubble increases, and the temperature can reach 10.0000 °C, causing the explosion of the bubble which liberates a large amount of energy, although for small distances (microns). The tissues and cells in the vicinity, accordingly, can be damaged.

The guide and monitoring through real-time imaging ensures that the FUS beam is focused on the area during the procedure and MRI imaging modalities are currently used for driving and monitoring the FUS treatment.

The combination of high-intensity focused ultrasound with high-resolution MR guidance has created a system able to produce the destruction of the tissues deep within solid organs, without any invasion. MRI allows precise targeting of ultrasound and also provides an accurate real-time thermal mapping. Nanocarriers and microbubbles can also be used for drug delivery because they provide a better contrast.

In fact, using nanocarriers sensitive to mechanical forces and/or sensitive to temperature, the contents can be released locally.

Thermosensitive liposomes for drug delivery have been proposed more than 30 years ago, when Yatvin *et al.* in 1978 described the effect of hyperthermia on the liposomal carrier.[91]

But only in 2006, it has been introduced the use of liposomes in combination with HIFU. In particular, doxorubicin loaded liposomal particles (Doxil) in combination with HIFU have been used in a mouse model of breast cancer.[92] In this particular study, the doxorubicin loaded liposomal particles did not reach a therapeutic advantage since Doxil was not thermosensitive; however, it was shown that HIFU induced an increase in the permeability of macromolecules in tumors.

The following year the first study on thermosensitive liposomes and HIFU was presented.[93] The same team then developed thermosensitive liposomes MRI-labeled to induce liposomes to release the drug locally and to improve therapeutic profiles.

A further application of HIFU was the combination with microbubbles to enhance the permeability of bioactive molecules across the blood-brain barrier (BBB). In a 2002 study, it was observed that the HIFU can alter the BBB, inducing a reversible disturbance in a targeted area,[94] and microscopy showed that the architecture of the brain was completely maintained after treatment.

The MRI-guided FUS method could be used for various therapeutic applications. For example, for the therapeutic delivery of large molecules that do not permeate the BBB or to deliver magnetic nanoparticles of iron oxide (MNPs) conjugated with an antineoplastic agent such as epirubicin.[95] The MNPs when delivered with FUS have favorable characteristics to MR imaging, demonstrating considerable accumulation in the brain of rats of both the MN particles and epirubicin.

The same method could also be used for transferring exogenous genes in the central nervous system, or for delivering therapeutic stem cells for the treatment of neurodegenerative diseases, brain injury, and stroke.

This strategy for drug delivery is a promising clinical approach and can be translated to the clinic as Magnetic Resonance-guided Focused Ultrasound (MRgFUS). The main delivery vehicles described until now are thermosensitive liposomes and microbubbles, but new research should aim to use other nanocarriers like micelles, because the combination of micelle with FUS could substantially enhance their delivery in target tissues. Nonetheless, the biological effects of FUS in the presence or absence of carriers will require more thor-

ough examination, in particular regarding the long-term effects on normal tissues to be evaluated in preclinical studies to design a safe and effective FUS drug treatment regimens.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.
Article first published online: November 18, 2016. - Manuscript accepted: November 17, 2016. - Manuscript received: October 3, 2016.