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Review Article

DIAGNOSTIC IMAGING PATHWAYS: ULTRASOUND CONTRAST AGENTS

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Abstract: Ultrasonography lacked substances to be administered to patients to improve or increase the diagnostic yield, which is peculiar considering that contrast agents have long been used with all the other imaging techniques. Fortunately some contrast agents, most of them consisting in gas microbubbles, have been recently introduced for ultrasound imaging too: this review will focus on their history, behavior, current applications and future developments. No definitive conclusions can be drawn yet on the actual merits of each contrast agent, but all of them seem to be both effective and safe, meaning that their future success will depend on the relative cost-effectiveness and peculiarities.

Key Words: Ultrasonography, Contrast-enhanced ultrasound (CEUS), Microbubbles, Diagnostic Imaging Pathways

Introduction: Contrast-enhanced ultrasound (CEUS) involves the administration of intravenous contrast agents containing microbubbles of perfluorocarbon or nitrogen gas. The bubbles greatly affect ultrasound backscatter and increase vascular contrast in a similar manner to intravenous contrast agents used in CT and MRI. Examples of ultrasound contrast agents available commercially include SonoVue (Bracco).

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CEUS has the advantage over contrast-enhanced MRI and CT in patients with contraindications such as renal failure or contrast allergy[2]. CEUS also allows for dynamic and repeat examinations[1].

An individual microbubble is estimated to measure approximately 6 micrometers, compared to a human erythrocyte measuring approximately 9 micrometers. Microbubbles are therefore not filtered in the lungs since they are equivalent in size to red blood cells. Microbubbles are different than the agitated saline used in echocardiographic "bubble studies". US contrast agents (UCA) consist of microscopic bubbles of gas enclosed in thin flexible shell. The types of gas and shell material used differ depending on the brand of

contrast agent. The microbubbles are generally 1-4 micrometers in size (smaller than a red blood cell) making them small enough to flow easily through the circulation, but large enough so they remain inside the blood vessels. An individual microbubble is estimated to measure approximately 6 micrometers, compared to a human erythrocyte measuring approximately 9 micrometers. Microbubbles are therefore not filtered in the lungs since they are equivalent in size to red blood cells. Microbubbles are different than the agitated saline used in echocardiographic "bubble studies"[3].

Depending on their composition, injection method and dose, microbubbles can be detected in circulation from several minutes up to 60 minutes, after which their gas core diffuses out of the shell and the components are cleared by the reticuloendothelial system. When high frequency sound waves from an ultrasound probe hit them, they oscillate and reflect a non-characteristic echo. The ultrasound probe is directed to send a special pulse inversion signal which enhances the echoes from the UCA, while reducing the echoes from the surround tissuing. This results in an enhanced image of the tissue vasculature. The UCA may continue to oscillate for a short time before it bursts. The gas diffuses into the bloodstream, and the shell material is metabolised. Generally the UCA is given as an injection and only lasts a short time in the body[4]. If longer times are required, UCA may be given through a drip to maintain a steady infusion of contrast.

Microbubble contrast agents have been used in ultrasound imaging as a means of improving the visualization of blood flow with respect to the surrounding tissue. These microbubbles can either be Non-targeted for visualization of blood flow and perfusion, or conjugated to a molecular target **(**Target-Ready**)** to enable true in vivo molecular imaging. Ultrasound contrast agents offer high sensitivity (the ability to ‘see' a single bubble) with a safety profile that is at least as good as conventional contrast agents. CEUS offers several advantages over the alternative imaging modalities[5].

With the introduction of microbubble contrast agents, diagnostic ultrasound has entered a new era that allows the dynamic detection of tissue flow of both the macro and microvasculature. Underpinning this development is the fact that gases are compressible, and thus the microbubbles expand and contract in the alternating pressure waves of the ultrasound beam, while tissue is almost incompressible. Special software using multiple pulse sequences separates these signals from those of tissue and displays them as an overlay or on a split screen. This can be done at low acoustic pressures (MI < 0.3) so that the microbubbles are not destroyed and scanning can continue in real time[6].

The clinical roles of contrast enhanced ultrasound scanning are expanding rapidly. They are established in echocardiography to improve endocardial border detection and are being developed for myocardial perfusion. In radiology, the most important application is the liver, especially for focal disease. The approach parallels that of dynamic CT or MRI but ultrasound has the advantages of high spatial and temporal resolution. Thus, small lesions that can be indeterminate on CT can often be studied with ultrasound, and situations where the flow is very rapid (e.g., focal nodular hyperplasia where the first few seconds of arterial perfusion may be critical to making the diagnosis) are readily studied[7]. Microbubbles linger in the extensive sinusoidal space of normal liver for several minutes whereas they wash out rapidly from metastases, which have a low vascular volume and thus appear as filling defects. The method has been shown to be as sensitive as three-phase CT.

Microbubbles have clinical uses in many other applications where knowledge of the microcirculation is important (the macrocirculation can usually be assessed adequately using conventional Doppler though there are a few important situations where the

signal boost given by microbubbles is useful, e.g., transcranial Doppler for evaluating vasospasm after subarachnoid haemorrhage). An important situation where demonstrating tissue devitalisation is important is in interstitial ablation of focal liver lesions: using microbubble contrast agents at the end of a procedure allows immediate evaluation of the adequacy of the ablation which can be extended if needed; this is much more convenient and cost-saving than moving the patient to CT and perhaps needing an additional ablation session at a later date.

Similar considerations suggest that contrast-enhanced ultrasound might have a role in abdominal trauma: injury to the liver, spleen and kidneys can be assessed rapidly and repeatedly if necessary. Its role here alongside dynamic CT remains to be evaluated. Infarcts or ischaemia and regions of abnormal vascularity, especially in malignancies, in the kidneys and spleen seem to be useful and improved detection of the neovascularisation of ovarian carcinomas is promising. Similar benefits in the head-and-neck and in the skin while the demonstration of the neovascularisation of atheromatous plaques and of aggressive joint inflammation offer interesting potentials.

Ultrasonography lacked substances to be administered to patients to improve or increase the diagnostic yield, which is peculiar considering that contrast agents have long been used with all the other imaging techniques. Fortunately some contrast agents, most of them consisting in gas microbubbles, have been recently introduced for ultrasound imaging too: this review will focus on their history, behavior, current applications and future developments. Echocontrast agent research is in progress and many new agents are expected to be marketed this and next year, to be added to Levovist by Schering AG (Berlin, Germany), to enhance the ultrasound signal safely and effectively. No definitive conclusions can be drawn yet on the actual merits of each contrast agent, but all of them seem to be both effective and safe, meaning that their future success will depend on the relative cost-effectiveness and peculiarities[9].

The basic principles of echocontrast agents: The microbubbles act as echo-enhancers by basically the same mechanism as that determining echo-scattering in all the other cases of diagnostic ultrasound, namely that the backscattering echo intensity is proportional to the change in acoustic impedance between the blood and the gas making the bubbles. The different acoustic impedance at this interface is very high and in fact all of the incident sound is reflected, even though not all of it will of course go back to the transducer. But the acoustic wave reflection, though nearly complete, would not be sufficient to determine a strong US enhancement because the microbubbles are very small and are sparse in the circulation. Moreover, reflectivity is proportional to the fourth power of a particle diameter but also directly proportional to the concentration of the particles themselves.

Second harmonic imaging: As we said above, the microbubbles reached by an ultrasound signal resonate with a specific frequency depending on microbubble diameter. However, the main resonance frequency is not the only resonance frequency of the bubble itself and multiple frequencies of the fundamental one are emitted, just like in a musical instrument. These harmonic frequencies have decreasing intensity, but the second frequency, known as the second harmonic, is still strong enough to be used for diagnostic purposes. The theoretical advantage of the harmonic over the fundamental frequency is that only contrast agent microbubbles resonate with harmonic frequencies, while adjacent tissues do not resonate, or else their harmonic resonation is very little. Thus, using a unit especially set to produce ultrasounds at a given frequency (3.5 MHz) and receive an ultrasound signal twice as powerful (7 MHz) it will be possible to show the contrast agent only, without any artifact from the surrounding anatomical structures, with a markedly improved signal-to-noise ratio[10].

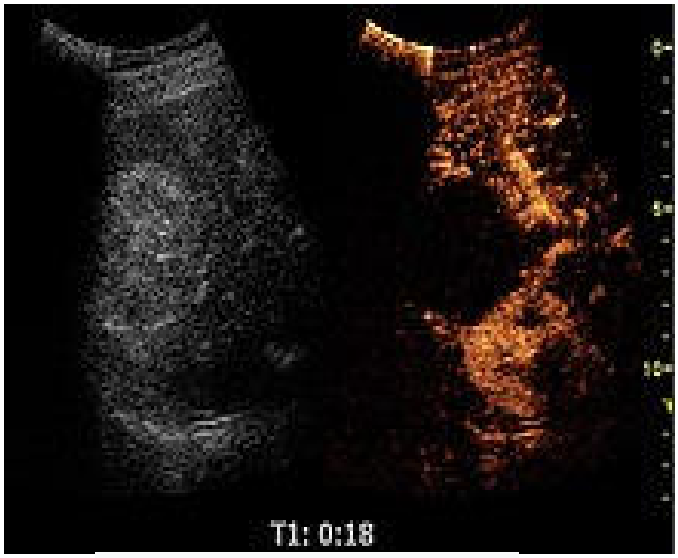

Figure 1. Contrast-enhanced renal ultrasonograph, showing a renal cell carcinoma successfully treated with thermal ablation, as no contrast enhancement is seen.

A similar effect to digital subtraction in angiography can thus be obtained, even though through a totally different process. Moreover, second harmonic imaging permits to show extremely small vessels (down to 40 microm) with very slow flow, which would be missed with a conventional method. B-mode imaging can also depict the microbubbles in the myocardium suppressing nearly all the artifacts from cardiac muscle motion. Recently a peculiar behavior of microbubbles has been observed which may permit contrast agent detection even in capillaries. This method is variously known as sonoscintigraphy, loss of correlation, stimulated acoustic emission and transient scattering[11]. The contrast agent microbubbles reached by an ultrasound beam powerful enough explode producing a strong and very short backscatter echo which is read by the unit as a Doppler signal and results in a color pixel where the individual microbubble exploded.

General features: There are a variety of microbubble contrast agents. Microbubbles differ in their shell makeup, gas core makeup, and whether or not they are targeted.

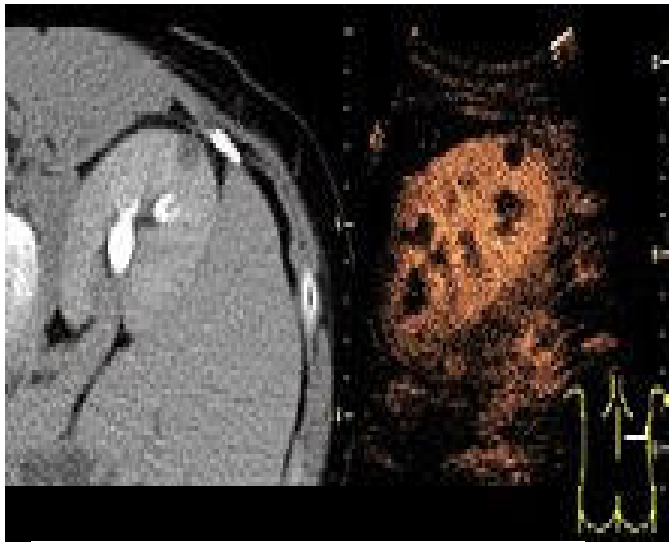

Figure 2. Unspecific cortical lesion on CT is confirmed cystic and benign with contrast-enhanced renal ultrasonography using image fusion.

Micro bubble shell: selection of shell material determines how easily the microbubble is taken up by the immune system. A more hydrophilic material tends to be taken up more easily, which reduces the microbubble residence time in the circulation. This reduces the time available for contrast imaging. The shell material also affects microbubble mechanical elasticity. The more elastic the material, the more acoustic energy it can withstand before bursting. Currently, microbubble shells are composed of albumin, galactose, lipid, or polymers.

Microbubble gas core: The gas core is the most important part of the ultrasound contrast microbubble because it determines the echogenicity. When gas bubbles are caught in an ultrasonic frequency field, they compress, oscillate, and reflect a characteristic echo- this generates the strong and unique sonogram in contrast-enhanced ultrasound. Gas cores can be composed of air, or heavy gases like perfluorocarbon, or nitrogen.[4] Heavy gases are less water-soluble so they are less likely to leak out from the microbubble leading to microbubble dissolution.[3] As a result, microbubbles with

heavy gas cores last longer in circulation. Regardless of the shell or gas core composition, microbubble size is fairly uniform. They lie within a range of 1-4 micrometres in diameter[12]. That makes them smaller than red blood cells, which allows them to flow easily through the circulation as well as the microcirculation.

How it works?

There are two forms of contrast-enhanced ultrasound, untargeted (used in the clinic today) and targeted (under preclinical development). The two methods slightly differ from each other.

Untargeted CEUS: Untargeted microbubbles, such as the aforementioned SonoVue, Optison, or Levovist, are injected intravenously into the systemic circulation in a small bolus. The microbubbles will remain in the systemic circulation for a certain period of time. During that time, ultrasound waves are directed on the area of interest. When microbubbles in the blood flow past the imaging window, the microbubbles' compressible gas
cores oscillate in response to the high frequency sonic energy field, as described in the ultrasound article. The microbubbles reflect a unique echo that stands in stark contrast to the surrounding tissue due to the orders of magnitude mismatch between microbubble and tissue echogenicity. The ultrasound system converts the strong echogenicity into a contrast-enhanced image of the area of interest. In this way, the bloodstream's echo is enhanced, thus allowing the clinician to distinguish blood from surrounding tissues.

Targeted CEUS: Targeted contrast-enhanced ultrasound works in a similar fashion, with a few alterations. Microbubbles targeted with ligands that bind certain molecular markers that are expressed by the area of imaging interest are still injected systemically in a small bolus. Microbubbles theoretically travel through the circulatory system, eventually finding their respective targets and binding specifically. Ultrasound waves can then be directed on the area of interest. The targeted microbubbles also reflect a unique echo that stands in stark contrast to the surrounding tissue due to the orders of magnitude mismatch between microbubble and tissue echogenicity. The ultrasound system converts the strong echogenicity into a contrast-enhanced image of the area of interest, revealing the location of the bound microbubbles.[8] Detection of bound microbubbles may then show that the area of interest is expressing that particular molecular marker, which can be indicative of a certain disease state, or identify particular cells in the area of interest[13].

Applications: Untargeted contrast-enhanced ultrasound is currently applied in echocardiography and radiology. Targeted contrast-enhanced ultrasound is being developed for a variety of medical applications.

Untargeted CEUS: Untargeted microbubbles like Optison and Levovist are currently used in echocardiography. In addition, SonoVue[9] ultrasound contrast agent is used in radiology for lesion characterization.

Organ Edge Delineation: Microbubbles can enhance the contrast at the interface between the tissue and blood. A clearer picture of this interface gives the clinician a better picture of the structure of an organ. Tissue structure is crucial in echocardiograms, where a thinning, thickening, or irregularity in the heart wall indicates a serious heart condition that requires either monitoring or treatment.

Blood Volume and Perfusion: Contrast-enhanced ultrasound holds the promise for (1) evaluating the degree of blood perfusion in an organ or area of interest and (2) evaluating the blood volume in an organ or area of interest. When used in conjunction with Doppler ultrasound, microbubbles can measure myocardial flow rate to diagnose valve problems. And the relative intensity of the microbubble echoes can also provide a quantitative estimate on blood volume.

Lesion Characterization: contrast-enhanced ultrasound plays a role in the differentiation between benign and malignant focal liver

lesions. This differentiation relies on the observation or processing of the dynamic vascular pattern in a lesion with respect to its surrounding tissue parenchyma[14].

Targeted CEUS

Inflammation: Contrast agents may be designed to bind to certain proteins that become expressed in inflammatory diseases such as Crohn's disease, atherosclerosis, and even heart attacks. Cells of interest in such cases are endothelial cells of blood vessels, and leukocytes: The inflamed blood vessels specifically express certain receptors, functioning as cell adhesion molecules, like VCAM-1, ICAM-1, E-selectin. If microbubbles are targeted with ligands that bind these molecules, they can be used in contrast echocardiography to detect the onset of inflammation. Early detection allows the design of better treatments. Attempts have been made to outfit microbubbles with monoclonal antibodies that bind P-selectin, ICAM-1, and VCAM-1, but the adhesion to the molecular target was poor and a large fraction of microbubbles that bound to the target rapidly detached, especially at high shear stresses of physiological relevance.

Leukocytes possess high adhesion efficiencies, partly due to a dual-ligand selectin-integrin cell arrest system. One ligand:receptor pair (PSGL-1:selectin) has a fast bond on-rate to slow the leukocyte and allows the second pair (integrin:immunoglobulin superfamily), which has a slower on-rate but slow off-rate to arrest the leukocyte, kinetically enhancing adhesion. Attempts have been made to make contrast agents bind to such ligands, with techniques such as dual-ligand targeting of distinct receptors to polymer microspheres, and biomimcry of the leukocyte's selectin-integrin cell arrest system, having shown an increased adhesion efficiency, but yet not efficient enough to allow clinical use of targeted contrast-enhanced ultrasound for inflammation[15].

Thrombosis and thrombolysis:

Activated platelets are major components of blood clots (thrombi). Microbubbles can be conjugated to a recombinant single-chain variable fragment specific for activated glycoprotein IIb/IIIa (GPIIb/IIIa), which is the most abundant platelet surface receptor. Despite the high shear stress at the thrombus area, the GPIIb/IIIa-targeted microbubbles will specifically bind to activated platelets and allow real-time molecular imaging of thrombosis, such as in myocardial infarction, as well as monitoring success or failure of pharmacological thrombolysis.

Cancer: Cancer cells also express a specific set of receptors, mainly receptors that encourage angiogenesis, or the growth of new blood vessels. If microbubbles are targeted with ligands that bind receptors like VEGF, they can non-invasively and specifically identify areas of cancers.

Gene Delivery: Vector DNA can be conjugated to the microbubbles. Microbubbles can be targeted with ligands that bind to receptors expressed by the cell type of interest. When the targeted microbubble accumulates at the cell surface with its DNA payload, ultrasound can be used to burst the microbubble. The force associated with the bursting may temporarily permeablize surrounding tissues and allow the DNA to more easily enter the cells.

Drug Delivery: drugs can be incorporated into the microbubble's lipid shell. The microbubble's large size relative to other drug delivery vehicles like liposome's may allow a greater amount of drug to be delivered per vehicle. By targeted the drug-loaded microbubble with ligands that bind to a specific cell type, microbubble will not only deliver the drug specifically, but can also provide verification that the drug is delivered if the area is imaged using ultrasound.

Safety:

Ultrasound contrast agents are generally tolerated very well and have a very good safety record. They are among the safest contrast

agents used in radiology. 1,2 The main serious adverse reaction is an anaphylactoid hypersensitivity reaction, which may occur in 1 in 7000 patients. This reaction is non-IgE mediated and may occur even if the patient has not been previously exposed to UCA. 2 However the overall rate of fatal events is quite low (approximately 1 in 500000). Less serious adverse reactions may include itching, moderate hypotension, headache, erythema, sensation of warmth and nausea & vomiting. 3 UCAs do not cause any renal impairment, and can be used in patients with any level of renal function[15].

A retrospective review of over 23000 injections of UCA in Italian centres found only 2 serious adverse reactions and no deaths (< 1 in 10000 serious adverse reactions). 3 There were 27 minor adverse reactions recorded (1 in 850). Another retrospective review compared the mortality & morbidity of 42000 patients who had UCA during rest & stress echocardiograms and compared with a matched cohort of 16000 patients who did not have UCA. They found that there were no significant differences in the rates of death of AMI. A smaller retrospective study looked for differences in adverse reaction rates in patients undergoing dobutamine stress echocardiography with & without UCA. 5 Two different types of UCA were used in a total of 1486 patients. The control group contained 1012 patients. They did not find any significant differences in the incidence of adverse events among the three groups.

It is recommended that any UCA administration should take place in the presence of an experienced clinician who is experienced in managing severe hypersensitivity reactions. Additionally, patients with pulmonary hypertension or unstable cardiopulmonary conditions should have continuous monitoring of their ECG & vital signs for at least 30 minutes.

Non-targeted contrast-enhanced ultrasound

More common method

- dynamic evaluation of the vascularity of a target lesion, most commonly in the liver or kidney, may be useful in diagnosis
- used to measure organ perfusion, which can be useful in diagnosing diffuse processes (e.g. cirrhosis)

Targeted contrast-enhanced ultrasound

Contrast agents designed to bind to specific molecules, which are then targeted at tissues expressing that substance.

Indications: Contrast-enhanced US has multiple and increasing uses including

- Cardiac US
- Characterisation of focal liver lesions
- Monitoring of percutaneous and transcatheter tumour ablation
- Vascular US
- Assessing vascularity of focal lesions (especially when CT and MR contrast agents are contra-indicated)

Ultrasound contrast agents composition

The lifetime of air bubbles is short. In 1968, Gramiak and Shah reported observations of clouds of bubbles after intra-aortic catheter injection of saline.[17] Further investigations reported on UCA consisting of saline, indocyanine green, hydrogen peroxide, dextrose, and renografin. Another approach was autologous blood injections at rapid rates which produced more stable bubbles. Neither gelatin nor agarose gel proved to be useful to stabilize bubbles. Synthetic polymers consisted of cyanoacrylate and air were marketed under the name of Sonovist®(Schering, Berlin, Germany). Those bubbles lasted more than 10 min and were taken up by the reticuloendothelial system. Other tested materials included poly(D, L-lactide-co-glycolide) and poly (vinyl-alcohol).

One of the first goals in producing effective UCAs around 1980 was to obtain stability long enough to reach the right heart. Since lung capillaries are efficient filters, it was not until the 1990s when left heart contrast became possible. Contrast-enhancing agents with improved stability to effectively enhance the blood pool appeared in 1995. The next objective

was to produce bubbles enabling real-time imaging. This goal was reached by replacing air with poorly soluble gases, e.g., perfluorocarbons, which improved bubble durability, along with sophisticated acoustic parameters enabling the development of software algorithms which could efficiently differentiate UCA from tissue signals.

All currently commercially available UCA consist of an inert gas encapsulated by a shell. The shell mainly influences the viscoelastic properties, i.e., stability and durability,[while the gas determines solubility and the majority of the bubbles' acoustic properties. As true blood pool agents, perfluorocarbons bubbles range from 1 to 10 μm in size, permitting passage through the pulmonary vascular system, which is essential for access to the systemic circulation. Soft shell materials consist of phospholipids or other surfactants and demonstrate improved nonlinear oscillations. Protein-shelled microbubbles are also available (e.g., Optison®) consisting of an albumin shell around perfluoropropane gas[18].

The terms "first and second generation UCA" are sometimes used to differentiate agents with air from those with low soluble gases. Although this overly simplifies the distinctions mentioned above, it is sufficient in clinical practice since development of second generation UCA leads to near complete disappearance of first generation agents due to the ease of use and effectiveness of the former. Uptake of microbubbles by Kupffer cells has been described in the liver and by macrophages outside of the liver. This mechanism depends on shell composition, size, and surface properties and cannot be predicted simply by the shell material[19]. The mechanism is understood for Levovist®, Sonazoid®, and Optison®. The extended late phase in Sonazoid® is termed the "post-vascular phase" where it may persist for several hours in the liver and spleen. So-called nanobubbles have inferior oscillation behavior relative to microbubbles but are of interest in therapeutic approaches; nanobubbles have a size of 400–800 nm and can extravasate into tumor tissue. The accumulation of nanobubbles in tumors is referred to as passive targeting.

Ultrasound Contrast Agents Imaging: UCA provide significant alterations in the reflection pattern. First, they increase the backscattered signal dramatically. When acoustic pressure is applied, UCA resonate in a linear manner. With increasing acoustic pressure, nonlinear vibrational patterns appear.[27] Tissue produces harmonic resonances only at higher mechanical index (MI), thus differentiation of signal origin whether tissue or UCA is possible[20]. Using filter systems, multiples of the natural frequencies are received, allowing a certain amount of background (non-UCA) signal suppression. High-pressure levels disrupt microbubbles producing powerful signals and signals of different qualities.

Bolus injection versus infusion: In most indications, UCA are simply injected as a bolus. Intravenous infusion is of interest in cardiac imaging and other indications. Wash-in and wash-out kinetics are produced with controlled destruction using US pulses with high MI. Higher rates of adverse events such as premature ventricular contractions in patients with coronary diseases have been reported after continuous injection.

UCA have been also used in physiological and nonphysiological (extravascular) body cavities. This is an off-label use except the application of Levovist® into the urinary bladder for evaluation of vesicoureteral reflux. Extravascular applications with SonoVue®, mentioned in the European Federation of Societies for US in Medicine and Biology guideline, include injection of contrast for assessment of hysterosalpingo-contrast-sonography, ascites to evaluate hepatic hydrothorax, bile ducts via percutaneous transhepatic cholangiography and drainage, endoscopic retrograde cholangiography or surgically placed T-tube, sialography, perianal fistula, abscesses, pseudocysts,

gastroesophageal reflux, Zenker's diverticulum, enema, and nephrostomy tubes[21].

How can we use ultrasound contrast agents imaging?

In cardiology, UCA are used to improve difficult echocardiograms. The frequency of difficult echocardiograms is given as approximately 30%. UCA imaging can improve these in a significant percentage. Cardiologic guidelines recommend UCA use in the following cases:

- If two contiguous segments of the left ventricular (LV) cavum are not observed
- To improve Doppler evaluations if the initial spectrum signals are inadequate
- If serial assessment of ejection fraction is required since UCA decrease variability
- If apical hypertrophic cardiomyopathy and noncompaction is suspected
- In the case of intracavitary thrombi, LV aneurysms, Takotsubo myopathy.

In most other indications, it is necessary to discriminate UCA from tissue signal as completely as possible.

Doppler-based methods: In high MI imaging, stimulated acoustic emission is used with color or power Doppler. The microbubbles are destroyed using a high MI US impulse and the received signal is a complex US wave mix resulting in a Doppler shift. It is particularly useful in the late phase of a UCA with tissue specificity, e.g., Levovist®. In EUS, color Doppler imaging has been used and it could be demonstrated that due to the small size of the image window real-time imaging was possible although spatial resolution was poor. Vascular recognition imaging represents a method in which advanced low MI contrast-specific imaging is mixed with a Doppler technique adding direction information in larger vessels.

Low mechanical index imaging and contrast-specific imaging: The optimal contrast-enhanced imaging method should provide high-resolution real-time imaging over a long period with B-mode information side by side or as an overlay to the UCA signal. Current imaging methods come close to that aim. Drawbacks are a varying degree of bubble destruction and a low quality of the fundamental (tissue) image and reduced local contrast resolution. Low MI techniques have two effects: First, bubbles do not burst and second, they elicit harmonic US waves. Reduced MI can lead to problems in depth penetration[22]. For providing specific UCA imaging, initially, a low-pass filter was used to remove the fundamental waves. The next evolution generating higher spatial resolution was the use of phase inversion modes with which the complete bandwidth of the transducer can be utilized. Here, phase inverted pulses are sent simultaneously which results in information = 0 when echoes are linear, and summing of information ≠ 0 when nonlinear echoes, such as UCA echoes reflect.

Why do we use ultrasound contrast agents imaging?

The described methods lead to separately displayed tissue and contrast signals. In general, the following questions can be answered.

Vital *versus* avital: The easiest way to use UCA is to differentiate enhancement *versus* nonenhancement. Due to the blood pool character and the high specificity of the signal, this is possible with high reliability.

In some clinical questions, neoplastic lesions and avital structures must be differentiated:

- Liver abscess, since pus frequently is not anechoic
- Intraductal papillary mucinous neoplasia with focal mucus accumulations which mimics neoplastic nodules
- Gallbladder stones without calcification which could be misdiagnosed as polyps
- Cardiac lesions mimicking thrombus but which are in fact neoplasms (unpublished data).

Detection of nonvascularized areas provides important information in characterizing many lesions. For example, focal nodular hyperplasia shows a central scar in about 2/3 of patients[58] while GI stromal tumors typically show necrotic

areas in contrast to lipoma, schwannoma, and leiomyoma.

General enhancement: Typically, the enhancement intensity of a lesion is compared with the surrounding reference tissue. Typical pancreatic ductal adenocarcinomas show a markedly lower degree of UCA uptake compared to surrounding pancreatic tissue in more than 90% of cases. In comparison, other entities, e.g., neuroendocrine tumors, lymphoma, metastases, and the "pseudosolid" entity "serous microcystic adenoma," typically show hyperenhancement. In daily practice, this discrimination from ductal adenocarcinoma is highly valuable due to the different approach in therapy and prognosis. In liver cirrhosis, regenerative nodules typically show enhancement similar to the surrounding liver tissue whereas hepatocellular carcinoma are hypervascular and, therefore, hyperenhancing in more than 90% of cases.

To differentiate atelectasis from lung neoplasia, the earliest appearance of UCA is significant. Early enhancement is indicative of atelectasis supplied by vasa communes, which lack the oxygen-rich blood demanded by neoplasms. Since vasa communes derive from the right ventricle, they enhance earlier than 7 s after injection while the vasa privata enhance later. This later enhancement pattern has a high probability for neoplasia but caution is required as infarcted lung can appear similarly.

Early phase – late phase: When UCA are injected as a bolus, the wash-in and wash-out kinetics can be evaluated. Analysis of the liver late phase allows reliable differentiation between benign and malignant focal liver lesions due to the hepatic dual blood supply. Lesions without portal veins show a shorter contrast enhancement resulting in a relative hypoenhancement about 30 s after injection. Such focal liver lesions are mostly malignant[23].

In the spleen, late phase hypoenhancement has a lower positive predictive value though for lesions with late phase enhancement similar to the surrounding tissue malignancy can virtually be ruled out. This principle is advantageous in daily routine when deciding which patients should be offered biopsy.

Special patterns: Specific patterns are shown by some tumor entities, e.g., centrifugal pattern of hepatic focal nodular hyperplasia and peripheral nodular enhancement in hemangioma.

Quantification: Enhancement kinetics are described elsewhere in this special issue of EUS.

Ultrasound Contrast Agents Safety: In October 2007, the Food and Drug Administration (FDA) issued a new product labeling for UCA due to four patient deaths and about 190 serious adverse events with unclear causation but association with UCA use. A "black box" warning regarding multiple disease state contraindications to UCA use was mandated, including acute myocardial infarction, decompensated heart failure, ventricular arrhythmias, or patients with high risk for the latter, respiratory failure, emphysema, conditions that may cause pulmonary hypertension and so on. Critics claimed that there was no proof for more than temporal relation, and cited the higher rates of incidents associated with alternative procedures after acute cardiac events. In 2008, the FDA downgraded the contraindications to warnings. Safety studies in around 200,000 patients receiving Optison® and Definity® demonstrated a very low adverse event rate. For Optison® and Definity®, there was an approximately 1:10,000 incidence of acute anaphylactoid reaction immediately after UCA injection.

Future Perspectives: Therapeutic applications of UCA include targeted thrombolysis and substance delivery. Examples are inducing thrombus dissolution in acute ST elevation myocardial infarction and intracranial thrombolysis. This has led to the development of micro- and nano-bubbles. Another example is sonoporation-induced drug delivery in patients with pancreatic cancer. An interesting contrast development is silica shell particles, which are

not actually bubbles, but be shown on high MI and could be useful as a therapeutic vehicle[23].

Targeting: Passive targeting refers to the tendency of microbubbles to accumulate in malignant lesions due to leaky vasculature and lack of lymph vessels draining the tissue. Active targeting requires surface modification, and the target must be presented on the luminal side of endothelial cells due to the blood pool character of microbubbles. *In vitro* studies and animal studies have been reported for models in thrombosis detection, atherogenesis, and transplant rejection. The diagnosis of tumors has been successfully shown in animals. Nevertheless, clinical studies have not been performed so far.

Therapeutic approaches: The concept of utilizing UCA to enhance the vibratory effects generated by US pulses has gained much attention. The research group around Tachibana demonstrated in 1995 that US thrombolysis is more effective in the presence of bubbles, which is explained by cavitation and other effects. US triggered substance delivery is based on the principle of destroying contrast bubbles by applying high energy US, which additionally increases capillary and cell membrane permeability. Microbubbles may also be used for gene therapy, but even with specially designed UCA, their effectiveness remains inferior to viral transfection modalities. Nevertheless, side effects of viral transfection are relevant and local control of UCA-controlled gene therapy is better. The principle of US-mediated drug release seems to be promising. Here, also sonoporation helps open the blood–brain barrier. Anti-tumor drugs could be delivered using UCA, but studies on animals have not reached the point where clinical trials seem to be close.

Conclusions: Microbubbles are used for contrast ultrasound imaging as blood-pool agents in cardiology and radiology. Their promise as targeted agents for molecular imaging is now being recognized. Microbubbles can be functionalized with ligand molecules that bind to molecular markers of disease. Potential clinical applications of molecular imaging with microbubble-based ultrasound contrast agents are in the monitoring of the biomarker status of vascular endothelium, visualizing tumor vasculature, and imaging inflammation and ischemia-reperfusion injury zones and thrombi. The microbubble contrast agents developed and introduced as safe and effective echo-enhancers in present-day clinical practice will open up new oppurtunities.

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