

Impact of the arterial input function on microvascularization parameter measurements using dynamic contrast-enhanced ultrasonography

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Abstract

AIM: To evaluate the sources of variation influencing the microvascularization parameters measured by dynamic contrast-enhanced ultrasonography (DCE-US).

METHODS: Firstly, we evaluated, *in vitro*, the impact of the manual repositioning of the ultrasound probe and the variations in flow rates. Experiments were conducted using a custom-made phantom setup simulating a tumor and its associated arterial input. Secondly, we evaluated, *in vivo*, the impact of multiple contrast agent injections and of examination day, as well as the influence of the size of region of interest (ROI) associated with the arterial input function (AIF). Experiments were

conducted on xenografted B16F10 female nude mice. For all of the experiments, an ultrasound scanner along with a linear transducer was used to perform pulse inversion imaging based on linear raw data throughout the experiments. Semi-quantitative and quantitative analyses were performed using two signal-processing methods.

RESULTS: *In vitro*, no microvascularization parameters, whether semi-quantitative or quantitative, were significantly correlated (P values from 0.059 to 0.860) with the repositioning of the probe. In addition, all semi-quantitative microvascularization parameters were correlated with the flow variation while only one quantitative parameter, the tumor blood flow, exhibited P value lower than 0.05 ($P = 0.004$). *In vivo*, multiple contrast agent injections had no significant impact (P values from 0.060 to 0.885) on microvascularization parameters. In addition, it was demonstrated that semi-quantitative microvascularization parameters were correlated with the tumor growth while among the quantitative parameters, only the tissue blood flow exhibited P value lower than 0.05 ($P = 0.015$). Based on these results, it was demonstrated that the ROI size of the AIF had significant influence on microvascularization parameters: in the context of larger arterial ROI (from $1.17 \pm 0.6 \text{ mm}^3$ to $3.65 \pm 0.3 \text{ mm}^3$), tumor blood flow and tumor blood volume were correlated with the tumor growth, exhibiting P values lower than 0.001.

CONCLUSION: AIF selection is an essential aspect of the deconvolution process to validate the quantitative DCE-US method.

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Key words: Dynamic contrast-enhanced ultrasonography; Angiogenesis; Linear raw data; Arterial input function; Functional imaging

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INTRODUCTION

Tumor angiogenesis is a process characterized by the development of new blood vessels supplying tumors with nutrients and oxygen^[1,2]. This process is an essential step for tumor growth as well as the initiation of metastasis. In this context, research is currently focused on developing anti-angiogenic treatments resulting in the destruction of neoblood vessels, which often occurs initially without any morphological changes^[3-5].

Since February 2000, Response Evaluation Criteria in Solid Tumors has been used by the entire cancer clinical research community as an essential tool in evaluating responses to anti-tumor treatments^[6]. As this involves analyzing morphological information^[7], it is commonly recognized that these criteria are no longer optimal in the early assessment of anti-angiogenic therapies, which primarily target microvasculature. Consequently, functional imaging is recognized as the best modality for evaluating such treatments.

However, the current microvascularization parameters on which therapy evaluations are based do not properly correlate with the vascular physiology, which might interfere with the final interpretation. Such parameters are defined as semi-quantitative since they only provide a relative access to the physiological parameters through the current ultrasound imaging mode. These limits may be overcome by working with the arterial input function (AIF), which takes into account physiological variations such as the cardiac flow rate, the arterial blood pressure, the vascular structure, and the way the contrast agent is injected^[8]. This is done through a deconvolution process which allows access to quantitatively-defined microvascularization parameters, since it provides absolute evaluation of microvasculature parameters. Deconvolution has been already performed using other imaging modalities^[9-11]. Previous studies have been performed to develop a deconvolution technique based on Tikhonov regularization^[12,13]: preliminary results demonstrated a diminution in microvascularization parameter coefficients of variation from 30% to 13%, respectively measured without and with the deconvolution process.

Considering these results, the aim of this study was to determine and analyze sources of variation that impact

microvascularization parameters measured using dynamic contrast-enhanced ultrasonography (DCE-US) combined with a deconvolution process, both *in vitro* and *in vivo*, following bolus injections of SonoVue® (Bracco, Milan, Italy).

MATERIALS AND METHODS

Contrast agent

DCE-US data were acquired following bolus injections of SonoVue®, a second generation echo contrast agent made of microbubbles filled with sulphur hexafluoride (SF₆) and stabilized by a shell of amphiphilic phospholipids^[14,15]. The diameter of the microbubbles, ranging from 1 to 10 μm^[15], allowed a purely intravascular circulation through the whole blood volume, which made it ideal for the evaluation of microvascularization^[15]. SF₆ is an innocuous, stable and inert gas that does not interact with any other molecules found in the body. In addition, SonoVue® is highly resistant to pressure changes and exhibits a non-linear response of the microbubbles when insonated at low acoustic power, thus provides continuous real-time ultrasonographic (US) imaging without any bubble destruction^[14,16,17]. SonoVue® is available as a kit which includes one vial of SF₆, a powder combining phospholipids and pharmaceutical grade polyethyleneglycol, and one syringe pre-filled with 5 mL of 0.9% sodium chloride. Before any US exam, reconstruction of the contrast agent was performed by introducing the content of the syringe into the vial followed by manual shaking for at least 20 s. After a few minutes of no use, the microbubbles tend to accumulate at the upper surface because of buoyancy. Thus, to inject a homogeneous bolus of contrast agent, the vial was manually checked before each injection. SonoVue® is stable for 6 h, and all experiments were conducted within this limit^[14].

Time intensity method

The time-intensity method is based on the dye-dilution theory, which provides a mathematical method for estimating microvascularization parameters based on monitoring the tracer concentration as a function of time generating a time intensity curve (TIC). Microvascularization parameters to be analyzed are extracted from the TIC^[18,19]. To be valid, a series of assumptions must be verified^[20]: (1) flow must be constant to ensure the amount of microbubbles injected has no effect on the flux; (2) blood and contrast agent must be mixed homogeneously; (3) recirculation should not interfere with the first pass; and (4) the mixing of the contrast agent must exhibit linearity and stability^[21]. In this context, linearity refers to the linear relationship between the injected concentration of contrast agents and the measured US signal. This was confirmed for low doses^[15] and in the context of bolus injections of contrast agent^[22].

In this study, these conditions were assumed to be satisfied, allowing the direct extraction of the microvascularization parameters from the TIC.

Ultrasound imaging mode

Images were acquired using a Toshiba Aplio® XG ultrasound scanner (Toshiba, Japan) connected to a 7-14 MHz linear transducer (PLT 1202S probe). (1) B-mode and Doppler imaging were performed at 14 MHz; and (2) Harmonic imaging was performed at 10 MHz using the pulse inversion mode in which two pulses were sent into the tissue, the second pulse being an inverted copy of the first one. Thus, the associated echoes were detected and summed^[23]. Linear scattering from the two pulses produced echoes that were inverted copies of each other, resulting in a null signal. Linear scattering dominates in tissue. Conversely, non-linear scattering of the two pulses, which dominates in microbubbles, produced echoes that were no longer inverted, resulting in a non-null signal^[24].

Data analysis

Semi-quantitative microvascularization parameters (the current method): Acquisitions and analyses were based on linear raw data (uncompressed data before standard video visualization). Such data exhibit the advantage of a linear dynamic range, which is the essential aspect of TIC analysis^[25,26]. Following bolus injections of SonoVue®, several harmonic images were acquired. Image analysis followed a protocol described in previous studies^[27,28] using dedicated software called CHIQ® (Toshiba). Thus, the current method allowed one to graphically extract semi-quantitative microvascularization parameters following the fitting of the tumor TIC based on minimizing the differences between the parameters of the raw curve and the coefficients of the IGR equation (Patent: WO/2008/053268 entitled “Method and system for quantification of tumoral vascularization”):

$$I(t) = a_0 + (a_1 - a_0) * \left(\frac{A + \left(\frac{t}{a_2}\right)^p}{B + \left(\frac{t}{a_2}\right)^q} \right)$$

$I(t)$ describes the variation in the intensity of contrast uptake as a function of time. a_0 is the intensity before the arrival of the contrast agent. a_1 is linked to the maximum value of contrast uptake. a_2 is linked to the rise time to the peak intensity (PI). p is a coefficient related to the increase in intensity. q is a coefficient related to the decrease in intensity. A and B are arbitrary parameters.

Semi-quantitative microvascularization parameters have already been described in previous studies^[27,28]. These are PI, the time to peak intensity (T_{PI}), the area under the curve (AUC), the wash-in (AUWI), the wash-out (AUWO) and the full width at half maximum (FWHM) (Figure 1^[12]).

Quantitative microvascularization parameters (the deconvolution process): Based on the dye-dilution theory, the influence of the arterial input manifests itself through the following fundamental convolution equation:

$$C_t(t) = \frac{\rho}{k_H} \cdot (BF) \cdot [C_a(t) \otimes R(t)]$$

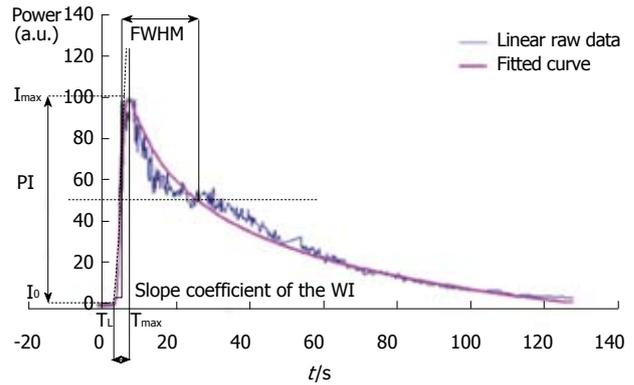


Figure 1 Graph displaying an example of a time intensity curve with four of its seven associated microvascularization parameters: the peak intensity, the time to peak intensity, the slope coefficient of the wash-in and the full width at half maximum. The three other microvascularization parameters extracted from the time intensity curve are the area under the curve, the area under the wash-in and the area under the wash-out. WI: Wash-in; FWHM: The full width at half maximum; PI: The peak intensity; I_{max} : Maximal intensity value; I_0 : Initial intensity value; T_L is the latency time and is defined as the time at the intersection between $y = I_0$ and the tangent at $T_{max}/2$ where T_{max} is the time associated with I_{max} .

where $C_t(t)$ is the concentration of the contrast agent measured in the tumor as a function of time, $C_a(t)$ is the AIF, BF is the tissue blood flow (unit: mL./min per 100 g of tissue), ρ is the tissue density (unit: g/mL) and k_H represents the difference in hematocrit between capillaries and arteries; ρ and k_H are commonly approximated by 1^[29,30]. $R(t)$ is the residue function and is defined as the relative amount of tracer in the region of interest (ROI) under the hypothesis of an instantaneous unit bolus injection [$R(0) = 1$; $R(\infty) = 0$]. The shape of the $R(t)$ function reflects the properties of both the vasculature and the tissue^[31].

In order to derive quantitative parameters from this equation, the residue function $R(t)$ can be determined using a process called deconvolution. The deconvolution method developed in our lab and dedicated to the DCE-US imaging is based on the Tikhonov regularization and was previously described by Gauthier *et al.*^[12].

Three quantitative microvascularization parameters are thus graphically extracted from the residue function: the BF, the tissue blood volume (BV) and the mean transit time (MTT) (Figure 2^[27]).

In vitro analysis

Three-intertwined pipe phantom: The phantom used in the *in vitro* experiments consisted of three-intertwined silicone pipes simulating a heterogeneous structure akin to that of vessels in tumor and feeding pipes which simulated the arterial input (Figure 3).

The phantom was immersed in a custom-made water tank connected to a peristaltic pump (SP vario/ PD 5101, Heidolph®, Germany) which provided a water flow rate set as required for the experiments. The three silicone pipes had an internal diameter of 2 mm with a 1 mm thick wall. Both the input and the output of the phantom were composed of three-way taps (Discifix®, B. Braun, Melsungen, Germany) allowing linkage between the three

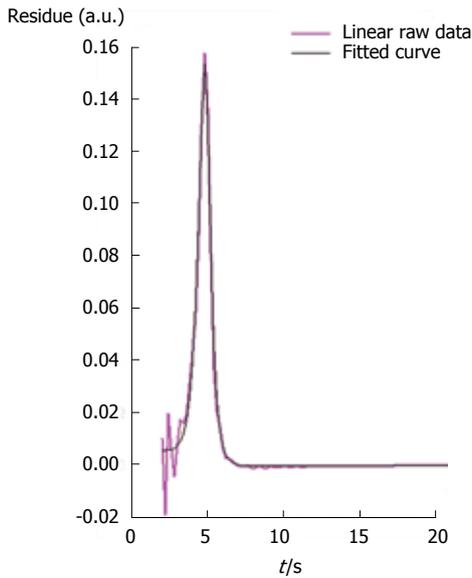


Figure 2 Graph displaying an example of a residue function. From this curve, three quantitative microvascularization parameters are graphically extracted: Blood flow corresponds to the maximum value reached by the residue function, blood volume is defined as the area under the curve and the mean transit time is defined as the ratio between blood volume and blood flow.

pipes. A new ROI, containing both pipes, feeding pipe and water spaces, was drawn for each injection and the associated TIC was obtained.

The total amount of water in the circuit was set at 60 mL. The amount of injected contrast agent was 0.06 mL. This volume corresponded to the ratio granted by French marketing approval (“Autorisation de Mise sur le Marché”: AMM) (2.4 mL of SonoVue® for 5 L of blood): this was defined as the AMM ratio. The second volume was associated with the ratio routinely used for clinical exams (4.8 mL of SonoVue® for 5 L of blood). In particular, this ratio was used in four studies led by the IGR involving 117 patients and 800 DCE-US exams^[32,33] as well as in a French national project supported by the “Institut National du Cancer” (French National Cancer Institute)^[34]. This ratio was defined as the internal growth rate ratio.

US protocol

Bolus injections of SonoVue® were performed using a 1 mL syringe (Terumo®, Belgium). To minimize potential errors due to SonoVue® residues, all of the injection materials were changed before each injection: the circuit was entirely emptied, rinsed and reset with water. Thus no contrast agent residues were present in the circuit, allowing the initial conditions to be exactly the same throughout all of the experiments^[27].

Before the series of acquisitions, a Doppler image was acquired to help the operator image the same setup through all the experiments.

Acquisitions were performed at a low mechanical index (MI = 0.21) and at a rate of 5 frames per second (fps). Two ROIs were selected to be analyzed (Figure 4^[12]): the first one was associated with the three-intertwined pipe

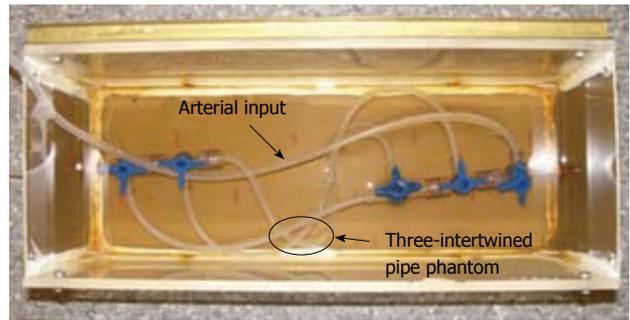


Figure 3 Three-intertwined silicone pipe phantom and its feeding pipe simulating the heterogeneous structure of a tumor and one of its arterial inputs.

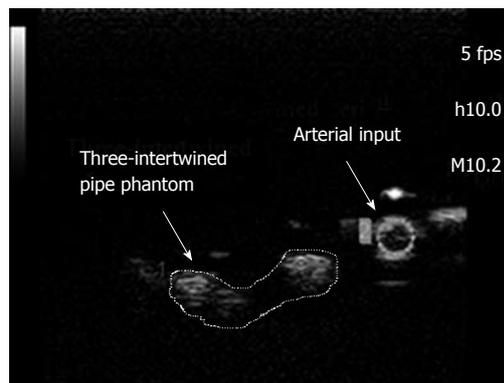


Figure 4 Pulse inversion image of the three-intertwined pipe phantom. The figure displays the selection of the region of interests associated with the phantom and its feeding pipe. fps: Frames per second.

phantom while the second was associated with its feeding pipe. Deconvolution was performed between the two TICs extracted from these ROIs through the CHIQ® software.

Repositioning of the US probe

Experimental protocol: Sources of DCE-US variability include the repositioning of the US probe before each examination: this has to be the same for each acquisition of the therapeutic evaluation process which can last several months or years. Indeed, evaluation of therapy is based on analyzing evolution of microvascularization parameters linked to a specific target along the whole duration of the treatment. In this context, the aim of this study was to evaluate the impact of the repositioning of the US probe on microvascularization parameters. The experiments consisted of: (1) 10 consecutive contrast agent injections with the US probe unchanged; and (2) 10 consecutive contrast agent injections with the US probe manually replaced before each exam.

Variation in flow

Experimental protocol: The absolute evaluation of the microvascularization parameters is not possible through the methodology which does not take into account the arterial input^[8]. Until now, different studies have investigated and used the link between the microvascularization



Figure 5 *In vivo* experiments were conducted on xenografted B16F10 female nude mice.

parameters directly extracted from the tumor TIC and the physiological parameters of interest^[35-38].

Thus, the BF is linked to the slope of the WI and the T_{PI} , which are currently the two parameters mentioned in the literature. None of the semi-quantitative microvascularization parameters are specific to the BF. On the contrary, as expected by definition, only one quantitative microvascularization parameter should be specific to the blood flow: it is BF.

To perform the study, 4 injections per flow rate were performed and 3 flow rates were tested: 21.2 mL/min, 42.4 mL/min and 63.6 mL/min. Variations in flow rates were controlled using the peristaltic pump.

***In vivo* analysis**

Animals and tumor model: Animal experiments were conducted with five nude female mice aged from six to eight weeks with the approval of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 18.III.1986; text amended according to the provisions of protocol ETS No. 170 as of its entry into force on 2nd December 2005). The tumor model B16F10 (CRL-6475, ATCC, American Type Culture Collection) melanoma cell line, a murine skin cancer, was selected to perform the *in vivo* experiments. Tumor cells were prepared and cultured in Dulbecco Minimum Essential Medium (Gibco Life Technologies, France) combined with 10% fetal bovine serum, 1% penicillin/streptomycin and glutamate (Invitrogen Life Technologies, Inc., France) to avoid bacterial contamination of the solution. While growing, cells were maintained in an incubator at 37 °C. Tumors were xenografted onto the right flank (Figure 5^[12,27]) of five mice through a subcutaneous injection of 2×10^6 melanoma cells in 0.2 mL of phosphate buffered saline. DCE-US exams were performed following three 0.1 mL injections of SonoVue[®] according to the methodology used in our lab.

Anesthesia

Mice received chemical anesthesia based upon their weight. Product was injected intraperitoneally using the 1 mL syringe. The solution consisted of ketamine (10 mg/mL,

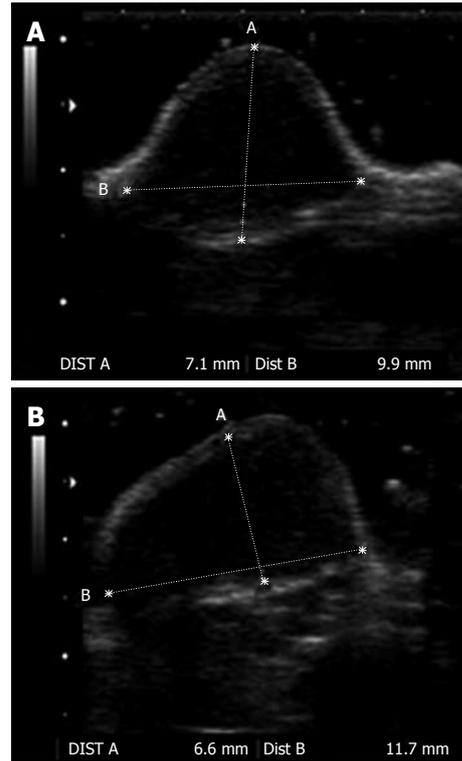


Figure 6 Volume evaluation was performed for each mouse using transversal (A) and longitudinal (B) B-mode images of the tumor.

Ketalar[®], Parapharm, France) and xylazine (2%, Rompum[®], Bayer, France). To ensure the mice remained asleep throughout the experiment, 150 μ L/g per mouse was systematically injected.

Acquisition protocol

In vivo images were acquired using the same ultrasound machine and probe as the *in vitro* experiments. Images were acquired using a mechanical index set at 0.21 and a rate of 5 fps. In addition, preliminary fundamental B-mode images were acquired to evaluate the tumor volume prior to the SonoVue[®] injection (Figure 6^[12]). The three perpendicular tumor diameters were determined and the tumor volume was derived according to the following formula:

$$V = \frac{1}{2} \times (\text{depth} \times \text{width} \times \text{length})$$

In the following study, two new ROIs were selected for each acquisition. The first ROI included only the tumor while the second ROI was associated with an arterial input. We gave preference to the location of the arterial ROI instead of its size to avoid any delay or dispersion effects that can impact the quantitative microvascularization parameters^[30]. Two TICs were extracted from these ROIs. To ensure the complete elimination of the contrast agent between each acquisition, a break of 15 min and 3 min of insonation at high MI (MI = 1.04) were observed. Mice were kept asleep no more than 2 h^[39]. This duration included the time required for the mice to obtain a stable

Table 1 Characteristics of the *in vivo* experiments

Mouse number	Number of days	Number of injections
Multiple injections and day of examination		
1	4	3
		4
		3
		4
2	2	3
		2
		4
3	2	2
		2
		4
Influence of the size of the arterial region of interest		
1	3	3
		3
		2
2	3	2
		3
		3
3	2	4
		3
		3
4	2	4
		3

heart rate after the administration of anesthesia, acquisition time and the time of the break between each injection.

Evaluated sources of variation

Multiple injections/day of examination: The first evaluated factor was the order of injection. This is a parameter of interest especially in the context of DCE-US exams that sometimes require the re-injection of the contrast agent. This study aimed to confirm that multiple injections had no significant impact on semi-quantitative and quantitative microvascularization parameters.

The second factor included different parameters. It was defined as the examination day to ensure no restriction of its meaning. Because melanoma cell lines exhibit a high doubling time of about 20 h^[40-42], the examination day may be a source of variation. Thus, to analyze this second factor amounts to evaluating whether any morphological or functional modification of the tumor significantly interferes with the microvascularization parameters.

For logistic reasons, a total of 3 mice were evaluated over 4 d following a maximum of 4 contrast agent injections performed during the study (Table 1).

Size of the arterial ROI

Until now, no consensus has existed concerning the ideal location to select the AIF. In the literature, the need to find a compromise between the size of the arterial input and its location with regards to the tumor is often highlighted. Indeed, to avoid any partial volume effects which affect the ROI, the AIF must be extracted from an artery exhibiting a diameter larger than the intrinsic resolution of the functional imaging technique used^[43,44]. On the other hand, an AIF measured too far from the tissue of interest causes additional delay and dispersion involving

wrong estimation of quantitative parameters, in particular, an underestimation of the BF and an overestimation of MTT^[8,44,45].

In the previous *in vivo* study, the arterial ROI was selected as close to the tumor as possible, whatever its size. In the second *in vivo* study (Table 1), the impact of the examination day on microvascularization parameters, giving preference in the selection of the arterial input to its size rather than its location, was evaluated.

Statistical analysis in vitro and in vivo

The aim of the first *in vitro* study was to determine whether there is an impact on the microvascularization parameters while repositioning the US probe during experiments. Statistical analysis was based on comparing the variances of both the series of 10 acquisitions with the US probe unchanged and replaced. To do so, a bilateral Fisher test was performed. It consisted of calculating:

$$F = \frac{s_1}{s_2}$$

where *s*₁ and *s*₂ are the variances of each of the series. The determined value of *F* was compared to the *F*-value with (*m* - 1)(*n*₂ - 1) degrees of freedom where *m*₁ and *m*₂ are the number of experiments performed in the two series of acquisitions (*m*₁ = *m*₂ = 10).

Results were significant for a *P*-value lower than 0.05.

The aim of the second *in vitro* study was to determine whether there was significant modification of the microvascularization parameters while the flow rate was modified. Thus, statistical analysis was based on comparing the means of microvascularization parameters for the three series of acquisitions. To do so, an analysis of variance (ANOVA) was performed between the 3 series of experimentations. Results were significant for a *P*-value lower than 0.05.

In vivo, statistical analyses were performed using the same multivariate ANOVA as the second *in vitro* study: the two parameters included in the process were the order of injection and the day of examination. Results were significant for *P*-value lower than 0.05.

RESULTS

In vitro

Repositioning of the US probe: *In vitro*, no microvascularization parameters, whether semi-quantitative or quantitative, were significantly correlated with the repositioning of the probe. Table 2 provides *P*-values associated with each of the microvascularization parameters.

Variation of the flow rate: No semi-quantitative microvascularization parameter was specific to the flow rate: none of the semi-quantitative parameters were correlated with the factor of interest. In addition, among the quantitative microvascularization parameter, only BF varied significantly with the flow rate: BF exhibited a *P*-value of 0.004. All the *P*-values are summarized in Table 2.

Table 2 *In vitro* and *in vivo* experiments: Statistical analyses

	<i>In vitro</i> results		<i>In vivo</i> results	
	Repositioning of the probe: Fisher's test <i>P</i> values	Variation of the flow rate: ANOVA <i>P</i> values	Multiple injections: ANOVA <i>P</i> values	Day of examination: ANOVA <i>P</i> values
Semi-quantitative microvascularization parameters				
PI	0.077	< 0.001	0.080	< 0.001
T _{PI}	0.302	0.004	0.595	0.641
Slope of the WI	0.059	0.006	0.141	< 0.001
MTT	0.562	< 0.001	0.885	0.016
AUC	0.731	< 0.001	0.073	0.01
AUWI	0.276	0.024	0.060	0.001
AUWO	0.860	0.006	0.078	0.016
Quantitative microvascularization parameters				
BF	0.055	0.004	0.071	0.116
BV	0.063	0.053	0.429	0.015
MTT	0.131	0.649	0.126	0.645

ANOVA: Analysis of variance; PI: Peak intensity; T_{PI}: Time to peak intensity; WI: The wash-in; MTT: The mean transit time; AUC: The area under the curve; AUWI: The area under the wash-in; AUWO: The area under the wash-out; BF: The tissue blood flow; BV: The tissue blood volume.

Table 3 Areas of the arterial region of interests associated with the two *in vivo* studies described in the article

	Mean (mm ²)	SD	Min	Max
First <i>in vivo</i> study	1.17	0.6	0.4	2.3
Second <i>in vivo</i> study	3.65	0.3	3.5	4.1

SD: Standard deviation.

In vivo

Multiple injections/day of examination: Multiple injections had no significant impact on the microvascularization parameters. Indeed, no parameter exhibited *P*-value lower than 0.05. All the *P*-values are summarized in Table 2.

On the other hand, to perform therapy evaluation, it is expected to obtain microvascularization parameters varying significantly with the tumor growth: any modification in microvascularization would be detected through the analysis of the microvascularization parameters. In the study, it was demonstrated that semi-quantitative microvascularization parameters were correlated with the tumor growth while among the quantitative microvascularization parameter, only the BV was.

Size of the arterial ROI

The impact of the size of the arterial ROI on microvascularization parameters was evaluated according to the same protocol used in the previous *in vivo* study.

It was demonstrated that for larger arterial ROIs (from 1.17 ± 0.6 mm³ to 3.65 ± 0.3 mm³) (Table 3), all quantitative microvascularization parameters were correlated with the tumor growth. Table 4 summarizes all of the *P*-values associated with this study.

DISCUSSION

In vitro, it was demonstrated that semi-quantitative and quantitative microvascularization parameters were not significantly correlated with the repositioning of the US

Table 4 Influence of the size of the arterial region of interest (*in vivo* experiments: Statistical analyses)

Day of examination : <i>P</i> -ANOVA values	
Semi-quantitative microvascularization parameters	
PI	0.027
T _{PI}	0.010
Slope of the WI	0.070
MTT	< 0.001
AUC	0.808
AUWI	0.562
AUWO	0.832
Quantitative microvascularization parameters	
BF	< 0.001
BV	< 0.001
MTT	0.033

ANOVA: Analysis of variance; PI: Peak intensity; T_{PI}: Time to peak intensity; MTT: The mean transit time; AUC: The area under the curve; AUWI: The area under the wash-in; AUWO: The area under the wash-out; BF: The tissue blood flow; BV: The tissue blood volume.

probe before each acquisition. This is a promising result as in the context of therapy evaluation, the same target must be imaged during the entire treatment as evaluation is based on analyzing the microvascularization parameter evolution of a specific target through these exams. Results associated with the variation of the flow rates study were in agreement with the remarks mentioned before the experiments: all of the semi-quantitative microvascularization parameters were correlated with the flow rate while among the quantitative microvascularization parameters, only *BF* was specific to the flow rate.

In vivo, the importance of the size of the arterial ROI was shown through the first study: the arterial ROI must be large enough to allow the deconvolution process to be relevant in the context of therapy evaluation. This result is consistent with previous studies evaluating the importance of the arterial ROI selection^[9]. In particular, the partial volume effect on the AIF and its consequences on analyses have been reported in the literature^[46]. Here, the first results demonstrated significant correlation between

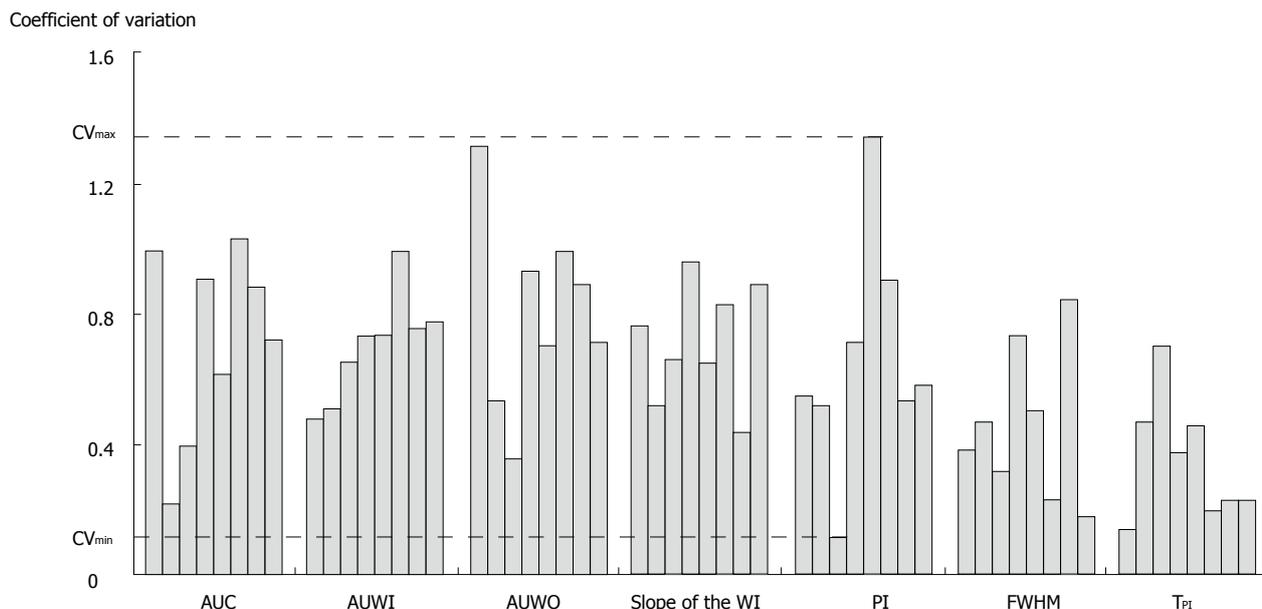


Figure 7 Display of the coefficients of variation, expressed as %, associated with the semi-quantitative microvascularization parameters extracted from the arterial region of interest. CV: Coefficient of variation; AUC: The area under the curve; WI: The wash-in; AUWI: The area under the wash-in; AUWO: The area under the wash-out; PI: Peak intensity; T_{PI}: Time to peak intensity; FWHM: The full width at half maximum.

semi-quantitative and quantitative microvascularization parameters except for T_{PI}, BF and MTT. In the context of experiments performed on mice, the T_{PI} and MTT were extremely short (in the order of a few seconds). Consequently, such low values make it difficult to distinguish modification in times linked to modification in microvascularization because of high statistical fluctuations. Previous studies showed the existing link between semi-quantitative microvascularization parameters and microvascularization quantitative ones. In particular, Lassen *et al*^[47] pointed out that the BF was related to the time to peak parameter. Thus, the same explanation may justify the absence of significance concerning this parameter. Further investigations have been performed to better interpret these first results. Coefficients of variation associated with the semi-quantitative microvascularization parameters extracted from the arterial ROI were evaluated (Figure 7). This evaluation provided a mean CV value of $0.63\% \pm 0.28\%$ which is high compared to previous intra-operator findings ($< 30\%$)^[27,48-53]. Such a result may be due to the very small arterial ROI prone to partial volume effects which induce noise in the deconvolution process, leading to the loss of correlation between quantitative microvascularization parameters and the tumor growth. Finally, the last experiments performed *in vivo* demonstrated significant correlation between quantitative microvascularization parameters and tumor growth for larger arterial ROIs. Identical results were obtained in the case of the semi-quantitative microvascularization parameters except for the areas where $P > 0.05$. One explanation may involve the high inter-subject variability noticed in the study. Thus, this high variability implies fluctuations in semi-quantitative microvascularization mean values that hide modifications in BV. On the other hand, as deconvolution allows freeing oneself from inter-

subject variability, results associated with the quantitative microvascularization parameters were significant.

Study limitations

In vitro, both the fluid and the pipes used did not exhibit the same ultrasound properties as blood and vessels, respectively^[54-56]. *In vivo*, quantification may have been difficult to perform because of the mouse's respiratory and cardiac movements which lead to instability in ROI locations. Another limitation involved the stability of the ultrasound contrast agent. Indeed, even if each experiment duration was less than 2 h on account of the stability of SonoVue[®] (6 h after its reconstitution as described by Schneider^[14]), recent studies have reported a significant incidence of spontaneous gas diffusion phenomena on temporal evolution of contrast microbubble size^[57-59]. This study did not take into account gas diffusion phenomena occurring for 2 h from initial formation of contrast agent. This assumption might have impacted the final results.

Further studies

The last *in vivo* study presented in the article was a preliminary study investigating the best selection of the arterial input. Further analysis must be performed to improve such a difficult selection. Indeed, Calamante *et al*^[60] have demonstrated that an arterial ROI not selected directly at the entrance of the tumor may lead to an underestimation of the BF and an overestimation of the MTT. Such wrong estimations were due to the delay and dispersion occurring between the site of injection and the arterial ROI. On the other hand, it may sometimes be difficult to access an arterial ROI large enough and in the close vicinity of the tumor leading to partial volume effect influencing the results^[16]. Thus, a trade-off between size and location of the arterial ROI must be determined.

Additional corrections to the deconvolution process may be required, as mentioned by van Osch *et al.*^[61], to ensure microvascularization parameters are accurately evaluated. Modeling of the AIF might also be necessary for the deconvolution process to be applicable in all cases^[62]. Finally, a proof of concept may be interesting in evaluating the robustness of the deconvolution method in a clinical context: additional studies may be performed to definitely validate the deconvolution process.

Advantage of the quantitative evaluation

As already mentioned, the current DCE-US method does not take into account the arterial input, which greatly depends on patient physiological conditions as well as on which contrast agent is injected. Parameters determined according to the method are graphically extracted from the tumor TIC and if studies demonstrated correlation between some of them and the physiological parameters, absolute quantification is not possible through that method. On the other hand, the deconvolution process allows absolute quantification providing clinically meaningful microvascularization parameters.

In addition, results presented in the article support the possible use of the deconvolution method in a similar context to the current DCE-US, as the last *in vivo* study exhibited identical results whatever the quantification process applied.

In the study, sources of variation that impact the microvascularization parameters measured using DCE-US were evaluated, both taking and not taking into account the arterial input.

Throughout all of the experiments, two main conclusions can be drawn. First of all, it is necessary to pay attention to the selection of the arterial ROI as a small ROI may be at the origin of noise in the deconvolution method leading to its impossible use as a quantification tool. Finally, when the arterial ROI is large enough, deconvolution exhibits similar results as the current DCE-US. Such results suggest that the deconvolution method may be useful in a similar context with the advantage of providing absolute quantification of the microvascularization.

COMMENTS

Background

The early and functional evaluation of new treatments in oncology, such as anti-angiogenic therapies, is a main goal. Today, dynamic contrast-enhanced ultrasonography (DCE-US) is commonly recognized as a functional imaging technique. It is a highly available and sensitive modality which allows early predicting of the tumor response to treatments involving changes in microvascularity before any morphological ones occur.

Research frontiers

Microbubble contrast agents for DCE-US have developed over the past 10 years and are currently approved in Europe, Asia and Canada. As it provides real time imaging, is widely available, non-ionizing, and low cost, ultrasound imaging is recognized as an ideal modality for angiogenesis. Recently, a deconvolution method dedicated to DCE-US imaging was developed to take into account the arterial input function which provides an absolute evaluation of microvasculature parameters. In this study, the authors aim to evaluate the sources of variation influencing the microvascularization parameters measured using

the routinely used DCE-US imaging method and the deconvolution method.

Innovations and breakthroughs

DCE-US is supported by the French National Cancer Institute which is currently studying the technique in several pathologies to establish the optimal microvascularization parameters and timing for quantitative anticancer efficacy assessments. This study firstly suggests the selection of the arterial region of interest (ROI) is important when using the deconvolution process, and secondly, when using the appropriate arterial ROI, analyses using deconvolution DCE-US and routine DCE-US exhibit similar results.

Applications

By comparing the deconvolution DCE-US and the routine DCE-US methods, this study may confirm the use of the deconvolution method in a similar context to the current routinely used DCE-US method. The deconvolution method exhibits the additional advantage of allowing absolute microvascularization quantification.

Terminology

DCE-US imaging involves the use of microbubble contrast agents and specialized imaging techniques to evaluate semi-quantitative microvascularization parameters. The deconvolution method is based on the Tikhonov regularization and aims to perform an absolute quantification of the microvascularization.

Peer review

This is part of an enthusiastic series of works, and is well organized. Other articles of the same authors are open. The end point of this study is clear: To evaluate the sources of variation influencing the microvascularization parameters measured by DCE-US. They performed an intensive experimental study using an *in vitro* and an *in vivo* model, and the results were clearly described. Even though a clinical situation may be further complicated, their study showed that their semi-quantitative microvascularization parameters might reflect reliable information of the nature of each parameter.

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