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LETTER

Caliper, contrast enhanced-ultrasound or laser speckle contrast imaging: Techniques to follow mice melanoma growth

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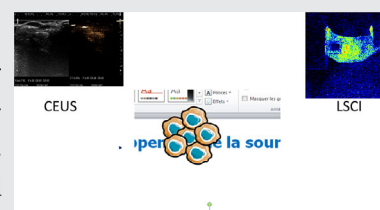
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Abstract

Due to morphological characteristics, metastatic melanoma is a cancer for which vascularization is not a diagnostic criterion. Laser speckle contrast imaging (LSCI) and contrast enhanced ultrasound (CEUS) are



two imaging techniques that will be explored in this study, which aims to confirm these two techniques for monitoring tumor vascularization. B16F10 cells were xenografted to C57BL/6 mice treated with anti-PD1 or 0.9% NaCl. Tumor volume was measured daily while CEUS and LSCI were performed weekly. LSCI and CEUS analyses showed a decrease in tumor perfusion in both groups of mice. Although both CEUS and LSCI are useful for measuring tumor volume, LSCI appears to be more robust and effective for monitoring tumor microcirculation. Non-invasive investigations are needed to better predict tumor vascularization: CEUS and LSCI have a good applicability in a mice model.

KEYWORDS

caliper, contrast enhanced ultrasound, laser speckle contrast imaging, melanoma, vascularization

INTRODUCTION

Cutaneous melanoma accounts for the bulk of melanoma diagnoses. Its incidence is ~25 new cases per 100 000 population in Europe, 30 cases per 100 000 population in the USA and 60 cases per 100 000 population in Australia and New Zealand [1]. Various strategies are being

considered to treat these melanomas, particularly metastatic forms, which are known to be aggressive and have a poor prognostic (15% 5-year survival rate). Among the molecules available, anti-PD1 immunotherapies restore antineoplastic immunity by limiting T cells exhaustion [2]. These new treatments have significantly modified the prognosis of metastatic or non-surgical melanoma with a 5-year survival about 50%, including side-effects and significant higher costs.

Adélie Mellinger and Jeanne Hersant contributed equally to this study.

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As with most solid tumors, melanoma vascularization is a major pathophysiological process in the growth of these tumors. Thus, angiogenesis, but also vascular mimicry [3] initially described by Maniotis et al. [4] are processes that have been widely described in recent years. Furthermore, at microcirculatory level, connection between endothelial and non-endothelial cells in extracellular matrix could increase tumoral cells exposure in blood flow.

The monitoring of tumor growth and assessment of treatment outcomes can be performed through various non- or less-invasive methods. While imaging modalities such as MRI or PET have been extensively studied, they present limitations such as temporal resolution [5, 6]. Photoacoustic imaging, which measures the conversion of electromagnetic energy into acoustic pressure waves, is combined with ultrasound and provides molecular information at clinically relevant depths with high real-time resolution [5]. Moreover, in the context of melanoma, photoacoustic imaging using melanin has demonstrated the ability to capture images of subcutaneous melanomas and their surrounding vascular structures in vivo [7, 8]. Dual-wavelength photoacoustic imaging can be employed to differentiate melanomas from tissue based on the optical absorption differences between hemoglobin and melanin [8]. Other imaging investigations to evaluate microcirculation should be considered such as the laser speckle contrast imaging (LSCI) and the contrast enhanced-ultrasound (CEUS).

The LSCI, with a good spatial resolution, allows a complete non-invasive screening of superficial tissue microvascularization perfusion. Laser light illuminates the measured tissue, and the resulting reflected light is captured by a measurement camera without direct contact with the tissue. Color-coded images are then generated from this signal, revealing the spatial distribution of tissue perfusion. Easy-to-use image analysis software (PIMSoft) assists in the evaluation of the results and in report generation (<https://www.perimed-instruments.com/content/pericam-psi-hr/>). This technic shows excellent intraobserver and interobserver reproductibilities [9,10]. Furthermore, the CEUS, with a high spatial and temporal resolution, is a noninvasive vascular investigation, which allows a good view of neoplastic tissues and have already been validated to evaluate tumor microcirculation in an animal model [11] and to vascular modification due to vascular mimicry [12].

In recent years, imaging techniques have been used to follow the growth of various tumors [13–15]. However, our study has evaluated the value of monitoring melanoma vascularization as a marker of tumor growth. Indeed, melanomas are generally tiny tumors, usually no more than 6 mm in diameter, for which it is not obvious to monitor growth, especially in animal models. This

study assumes that LSCI and CEUS would be two useful imaging techniques for following the growth of anti-PD1-treated melanoma, with particular attention paid to the degree of tumor vascularization.

MATERIALS AND METHODS

Cell culture

Mice melanoma cell line B16F10 were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (Lonza, Verviers, Belgium), 10 units of penicillin, 10 mg of streptomycin, 25 µg/mL of amphotericin B (Sigma-Aldrich, Saint-Louis, USA), and 1% nonessential amino acids (Lonza, Verviers, Belgium). Cell lines were cultured and maintained at 37°C in a humidified atmosphere with 5% CO₂.

In vivo experiments

All procedures involving animals, were conducted in accordance with protocols approved by ethical committee of the University of Angers and the regional ethics committee on animal experimentations (Authorization APAFIS#13365–2 018 020 217 041 961 v3). Furthermore, animal experiments were carried out in strict accordance with recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral research with Animals (European directive 2010/63/UE).

Seven-week-old male C57BL/six mice were housed at the university animal facility (Service Commun d'Animalerie Hospitalo-Universitaire–Université d'Angers, France). Syngenic allograft model of melanoma was obtained by injecting subcutaneously a suspension of 10⁵ B16F10 melanoma cells in 100 µL of PBS 1× (Lonza, Verviers, Belgium) into the right flank of mice. For optimal measurement of the tumor and its vascularisation, body hair was removed manually. The mice were shaved with a razor and the remaining hair was removed with depilatory cream. Tumor volume was monitored.

Mice were randomized and were treated when tumor volume was approximately 100 mm³. Mice were treated twice a week (days 1, 3, 8, 10, 15, and 18) with 3 mg/kg/day mice anti-PD1 (BioXcell, Lebanon, USA) or NaCl 0.9%. Tumor volume was monitored according to different techniques such as (i) caliper with the formula: $V = \pi/6 \times L \times W^2$ (V “volume”; L “length,” and W “width”) and (ii) ultrasonography. In accordance with ethical rules, animals were sacrificed when the tumor volume was greater than 2500 mm³ or when mice showed signs of suffering.

Contrast enhanced-ultrasound

This analysis was performed at the beginning of the protocol (W0) and after 1 week (W1). Mice were anesthetized with 2% isoflurane. Prewarmed echo transmission gel was applied to the tumor mice. Acquisition time did not exceed 80 s. For each tumor, at least three measures corresponding to length (L), width (w), and height (h), were made by ultrasonography imaging with an Arietta v70 equipment (Hitachi Medical Systems, Saint-Priest, France). The volume (V) in mm^3 was calculated considering tumors as semi-ellipsoids, with the formula $V = (4/3 \pi \times L \times w \times d)/2$ (w , L , and d , are the tumor width, length, and depth, respectively).

Laser speckle contrast analysis

Measurements were performed twice at W0 and W1 in an air-conditioned and temperature-controlled laboratory. LSCI measurements were performed by using a PeriCam PSI system and proprietary acquisition PIMSoft software. The wavelength used, as specified by the manufacturer, is the 785 nm laser diode (<https://www.perimed-instruments.com/content/pericam-psi-h/>). The distance between laser head and skin chosen by the operator was 12 cm. Acquisition time did not exceed 80 s. Mice were anesthetized with 2% isoflurane. Tumoral perfusion was evaluated in different ROI (region of interest)—one on an aluminum patch to avoid artefacts (ROI 1), one in normal skin (ROI 2), and one in tumoral site (ROI 3). Values of each ROI were expressed in Laser arbitrary perfusion units (LPU).

The perfusion was determined with the following equation: $(\text{ROI1}-\text{ROI3})/(\text{ROI2}-\text{ROI3})$.

Statistical analysis

Data were presented as means \pm SEM. Statistical differences were determined using unpaired Mann–Whitney U test when comparing between two independent groups, and Kruskal–Wallis test followed by a Dunn's post-hoc test when comparing across three or more independent groups. The p value <0.05 was considered significant. The tumoral growth analysis was performed thanks to a linear mixed model (longitudinal data), including the time influence (intraday and interday variability) and random intercept (intraindividual and interindividual variability). In order to obtain a reliable statistical model, the tumoral volume was converted into a decimal logarithmic scale. Survival analysis was done using a log-rank model to compare survival curves and to calculate survival medians.

RESULTS

Anti-PD1 antibody does not alter median or survival rate

No significant difference in mice survival was observed in anti-PD1 treated mice in comparison with control mice (Figure 1A). Moreover, no significant difference was observed in median survival: it was 12 days for control mice while 13 days for anti-PD1 treated mice (Figure 1B).

Measurement of tumor volume using a caliper or CEUS

Monitoring of tumor volume with caliper showed no significant difference between anti-PD1 treated mice and control mice (Figure 2A). Moreover, the measurement of tumor volume through CEUS did not show any difference between control and anti-PD1 treated mice at day 0 and after 7 days (Figure 2B). In control mice, analysis of tumor volume did not show any significant difference between caliper or CEUS techniques. Likewise, no significant difference was observed in anti-PD1 treated mice. However, although these data are not significant, the distribution of the data appeared to be different. Indeed, the group of mice treated with anti-PD1 and whose tumor volume was measured by caliper showed a wider dispersion of points, indicating greater variability and lower reproducibility for this condition. When tumor volume was measured by ultrasound, the distribution of points was tighter and more consistent, suggesting better reproducibility of the data (Figure 2C).

Better detection of microcirculation by LSCI than CEUS

From CEUS data, a difference in tumor size was observed at the beginning of the treatment in anti-PD1 treated mice in comparison with control mice. No difference was found after 1 week of treatment between these two groups (Figure 3A). LSCI analyses revealed a decrease in tumor perfusion after 7 days in anti-PD1 treated mice compared to control mice (Figure 3B).

An illustration of CEUS and LSCI assessment is presented in Figure 4.

DISCUSSION

Noninvasive imaging techniques are being developed and improved for identifying and evaluating pathophysiological

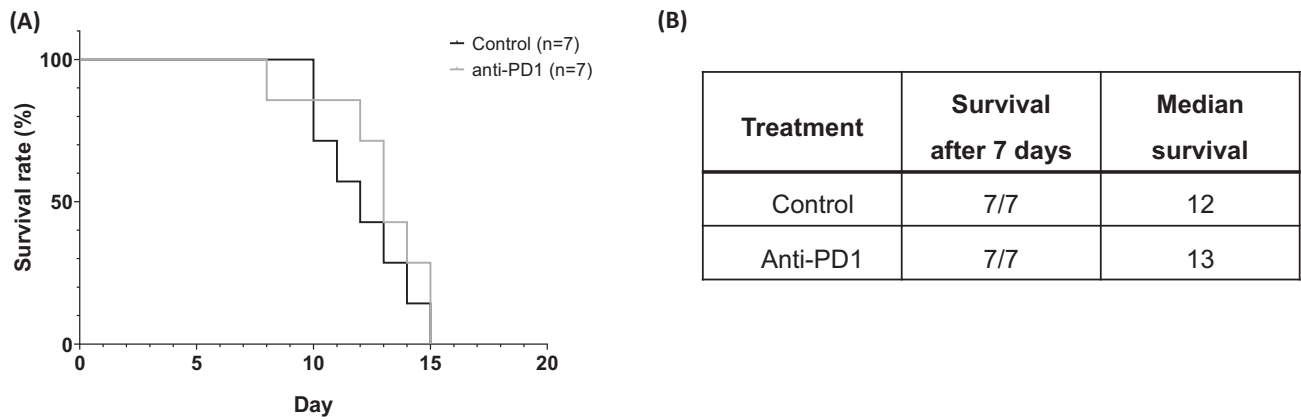


FIGURE 1 Survival of mice bearing B16F10-xenografted melanoma after treatment with mice anti-PD1 antibody or PBS. Treatment was initiated when the tumor volume was approximately 100 mm^3 . (A) Kaplan-Meier survival curve of treated or not mice. (B) Evaluation of mice survival after 7 days treatment, and the survival median after tumor xenograft ($n = 7$).

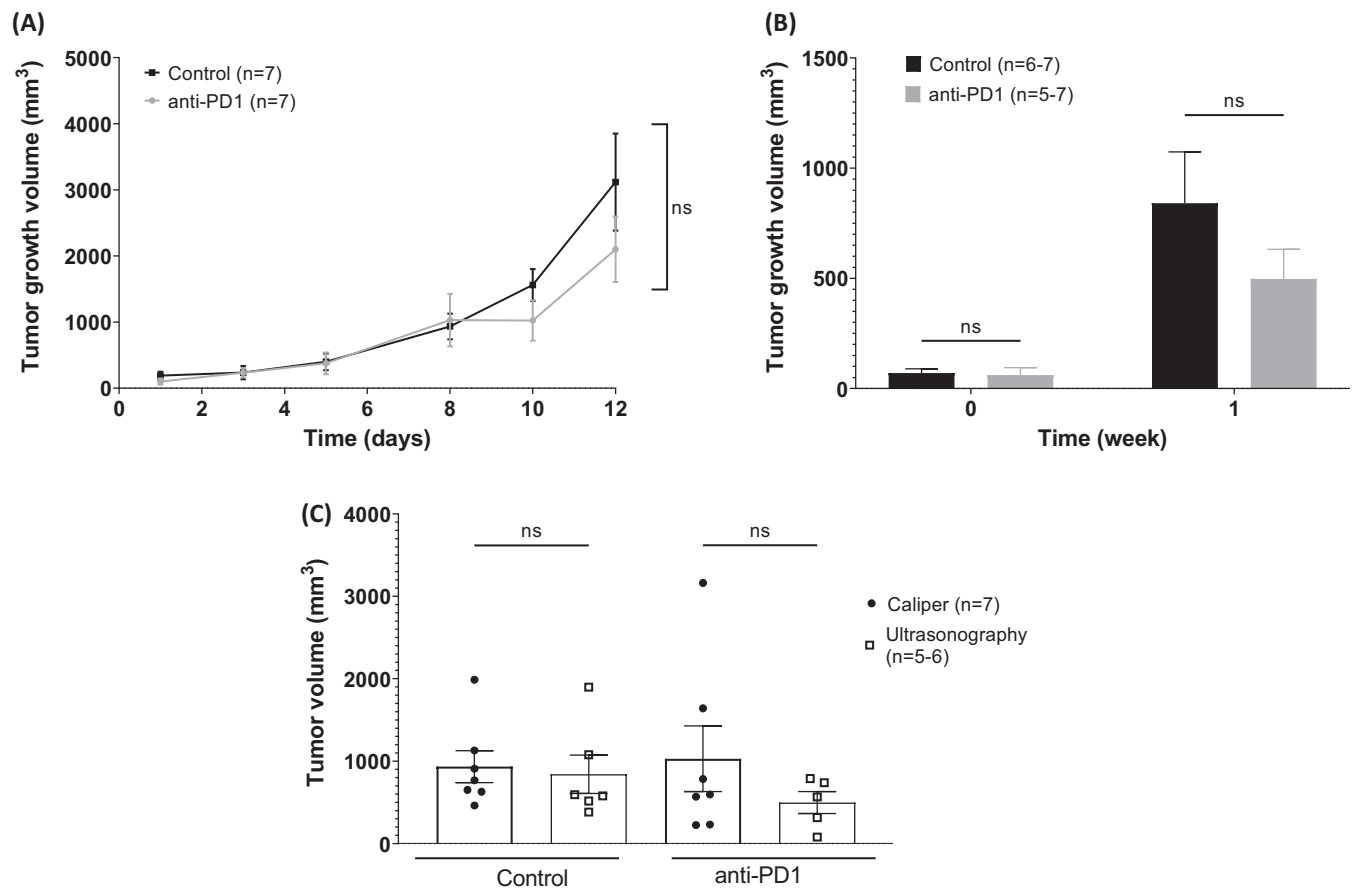


FIGURE 2 Evaluation of tumor volume from different methods in B16F10-xenografted mice treated with mice anti-PD1 or PBS. (A) Daily monitoring of tumor volume with caliper. (B) Evaluation of tumor volume through contrast enhanced ultrasonography (CEUS) at the beginning of the treatment and after 1 week of treatment. (C) Comparison of tumor volume monitoring by using caliper or through CEUS ($n = 5-7$). Data are expressed as the mean \pm SEM. Kruskal-Wallis test followed by a Dunn's post hoc test with a Hochberg correction and Mann-Whitney U test was used for statistical analysis (ns $p > 0.05$).

features of tumors in order to assist in the planning of individual patient treatment protocols [16,17]. Particularly, new imaging approaches that assess tumor

vascularization have improved diagnosis and treatment prediction. In this study, we evaluated (i) caliper and contrast enhanced ultrasonography (CEUS) to define

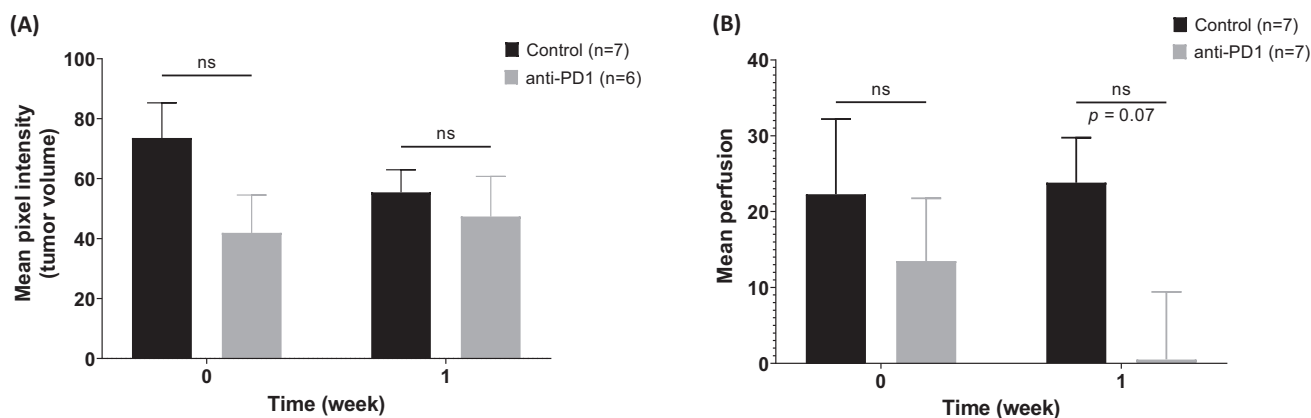


FIGURE 3 Evaluation of both tumor volume and perfusion from B16F10-xenografted mice treated with mice anti-PD1 or PBS. (A) Evaluation of tumor perfusion through contrast enhanced ultrasonography (CEUS) at the beginning of treatment and 1 week after. (B) Tumor perfusion was measured by laser speckle contrast imaging at the beginning of treatment and 1 week after. Data are expressed as the mean \pm SEM. Kruskal–Wallis test followed by a Dunn's post hoc test (ns $p > 0.05$).

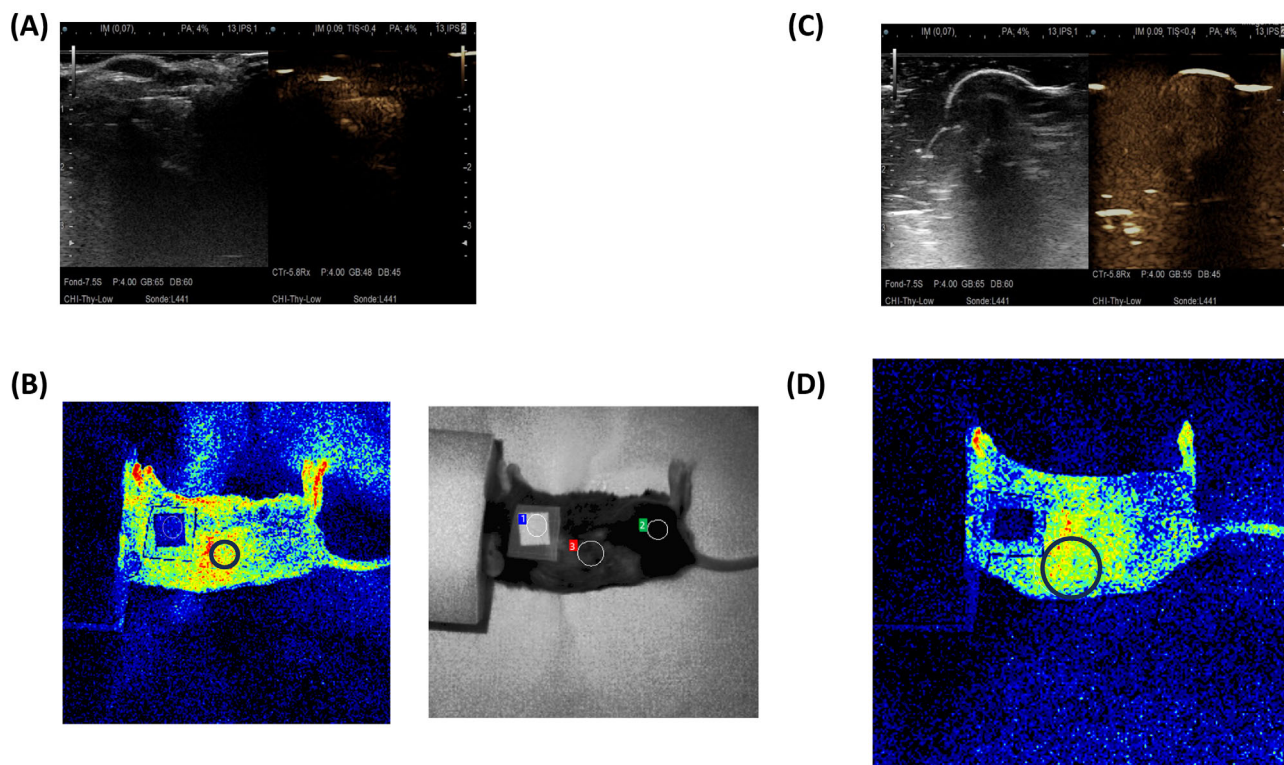


FIGURE 4 Contrast enhanced ultrasonography (CEUS) and laser speckle contrast imaging (LSCI) assessment on day 1 and day 7 in a control mice with B16F10-xenografted melanoma. (A) Day 1 CEUS assessment—volume measured at 40.6 mm³. (B) Day 1 LSCI investigation—ROI 1 (blue): aluminum patch; ROI 2 (green): normal skin; ROI 3 (red): tumoral skin (identified by a black circle); mean perfusion: 51 LPU. (C) Day 7 CEUS assessment—volume measured at 517.3 mm³. (D) Day 7 LSCI investigation—mean perfusion decreased to 26.95 LPU; tumoral Site identified by a black circle. CEUS requires direct skin contact, which can be challenging in case of superficial tumor, particularly in mice models, as illustrated in this figure. In contrast, LSCI provides a less operator-dependent assessment.

tumor dimensions and (ii) CEUS and LSCI to appreciate tumor vascularization.

The murine melanoma model is based on B16F10 cells known for their aggressiveness, high metastatic

potential, and high expression of the PD-1 receptor [18]. Taken together, these data confirm the value of evaluating the impact of anti-PD1 antibodies in limiting tumor growth. Although the survival data did not allow us to

conclude that there was a significant benefit from anti-PD1, they showed that before 7 days of treatment, no mortality was observed, whatever the group of animals, justifying the use of this time frame to evaluate the different imaging techniques.

To determine tumor dimensions, caliper, and CEUS were used in this study. Although tumor volumes are similar for both methods, as recently described in non-melanoma skin cancer [19], our data suggested an interest in CEUS because of various biases described with caliper. First, it has been described that caliper often overestimated tumor volume. Furthermore, the caliper measurements were smaller for small tumors compared to greater tumors also relatively seen. Consequently, volume changes measured with caliper in small and large tumors are not comparable and effects of anti-cancer drugs can easily be missed as tumors will tend toward being determined with a greater bias as they grow larger [20].

The value of CEUS has recently been confirmed by Makouei et al. [21]. In a pilot study conducted on mice with a soft tissue tumor, they confirmed that ultrasonography is a feasible and accurate imaging method to assess the tumor volume. However, these authors pointed out a number of limitations. Among these is the failure to take account of vascularization, which is an important factor in the diagnosis and treatment of metastatic tumors. To overcome these limitations, our study was supplemented by microcirculation analyses through LSCI in comparison with CEUS technique.

While CEUS analysis showed a reduction in microcirculation at treatment initiation, no difference was observed after 7 days of treatment suggesting that CEUS is not sufficiently robust for long-term studies. On the other hand, LSCI analysis confirmed a decrease in tumor perfusion over time in mice treated with anti-PD1 compared with control mice. Compared to CEUS, LSCI measurement did not need a direct skin contact, which is an advantage in these types of analyses. Indeed, a strongly hold of the ultrasound probe can create a local ischemia or reduce the microcirculatory vascularization and create biased results. Besides, LSCI measure results from local microvascular analysis without a blank analysis, which minimize artefacts. Last, if using the same experimental conditions, LSCI showed a good reliability and reproducibility. These data suggest the potential clinical value of LSCI in improving the diagnosis of metastatic melanoma.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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