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Welfare Assessment on Healthy and Tumor-Bearing Mice after Repeated Ultrasound Imaging

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Animal welfare · Ultrasound · Breast cancer · Severity assessment · Regorafenib · Isoflurane anesthesia

Abstract
Introduction: Ultrasound (US) imaging enables tissue visualization in high spatial resolution with short examination times. Thus, it is often applied in preclinical research. Diagnostic US, including contrast-enhanced US (CEUS), is considered to be well-tolerated by laboratory animals although no systematic study has been performed to confirm this claim. Therefore, the aim of this study was to screen for possible effects of US and CEUS examinations on welfare of healthy mice. Additionally, the potential influence of CEUS and molecular CEUS on well-being and therapy response to regorafenib was investigated in breast cancer-bearing mice. ***Material and Methods:*** Forty healthy Balb/c mice were randomly assigned for examination with US or CEUS (3×/week) for 4 weeks. Untreated healthy mice and mice receiving only isoflurane anesthesia served as controls (n = 10/group). Ninety-four 4T1 tumor-bearing Balb/c mice were allocated randomly to the following groups: no imaging, isoflurane anesthesia, CEUS, and molecular CEUS. They either received 10 mg/kg regorafenib or vehicle solution daily by oral gavage. Animals were examined three times within 2 weeks. CEUS measurements were performed using phospholipid microbubbles, and phospholipid microbubbles targeting the vascular endothelial growth factor receptor-2 were applied for molecular CEUS. Welfare evaluation was performed by daily observational score sheets, measuring the heart rate, Rotarod performance, and fecal corticosterone metabolites twice a week. On the last day, pathological changes in serum corticosterone concentrations, hemograms, and organ weights were obtained. Moreover, a potential influence of isoflurane anesthesia, CEUS, and molecular CEUS on regorafenib response in tumor-bearing mice was examined. Analysis of variance and Dunnett's post hoc test were performed as statistical analyses. ***Results:*** Severity parameters were not altered after repeated US and CEUS examinations of healthy mice, but spleen sizes were significantly lower after isoflurane anesthe-

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sia. In tumor-bearing mice, no effect on animal welfare after repeated CEUS and molecular CEUS could be observed. However, leukocyte counts and spleen weights of tumor-bearing mice were significantly lower in animals examined with CEUS and molecular CEUS compared to the control groups. This effect was not visible in regorafenib-treated animals. ***Conclusions:*** Repeated US and (molecular) CEUS have no detectable impact on animal welfare in healthy and tumor-bearing mice. However, CEUS and molecular CEUS in combination with isoflurane anesthesia might attenuate immunological processes in tumor-bearing animals and may consequently affect responses to antitumor therapy.

Introduction

In the past decades, noninvasive imaging has become an important tool in preclinical studies [1]. It is applied in various fields of basic research, such as oncology and drug development [2, 3]. In comparison to histological examinations, imaging methods offer the opportunity of performing longitudinal examinations of the same animal. This is strongly connected to the 3R (Reduction, Refinement, and Replacement) principles, by reducing the number of laboratory animals needed for one study. Moreover, longitudinal imaging contributes to the refinement of experiments by enabling an improved monitoring of the health state and disease progression of individuals, thus enabling better control of animal welfare [4]. In this context, ultrasound (US) imaging is frequently applied in preclinical research.

In humans, US imaging is regarded as safe when performed at a mechanical index (MI) below 1.9, a safety index that describes the risk to induce mechanical damage in tissue. It is calculated by dividing the US beam's peak negative pressure by the square root of its frequency [5]. Above an MI of 1.9, US may produce thermal or mechanical effects in tissues that can lead to changes in perfusion, cell necrosis, and apoptosis, depending on the applied US settings (e.g., frequency, pressure, pulse length, treatment time) [6, 7]. In animals, US is often performed at higher frequencies (human: 3–14 MHz; mouse: 16–70 MHz) to enable a sufficient spatial resolution in the considerably smaller organs, and imaging is applied more frequently, e.g., to monitor treatment effects in tumors.

Although mechanical effects are usually negligible in diagnostic US (MI <1.9), they may occur already below this safety threshold when US contrast agents (gas-filled microbubbles) are applied to investigate tissue vascularization. Microbubbles start to oscillate when they are stimulated by US, which produces very specific US signals that can be used to detect the microbubbles and discriminate them from the surrounding tissue. However, the oscillation can lead to shear stress on nearby cells due to acoustic microstreaming and a violent destruction of microbubbles produces shock waves [8]. As microbubbles remain intravascular based on their size of 2–3 μm, these effects are induced mainly on endothelial cells, and microvascular damage has been reported after contrast-enhanced US (CEUS) in rats mesentery and kidney [9, 10]. Furthermore, the surface of microbubbles can be coated with ligands (e.g., antibodies, peptides), which bind to specific components of the vascular system such as receptors expressed on endothelial or other cells exposed to the vascular lumen (e.g., platelets and immune cells) [11]. During molecular CEUS measurements, these receptor-bound microbubbles are often detected through destruction-replenishment measurements, where microbubbles are destroyed by short US pulses with high acoustic pressure. The signal of receptor-bound microbubbles can then be calculated by comparing the US signal before and after the destruction of microbubbles [11]. During their destruction, high temperatures or pressures are generated which can result in microjets or the production of reactive oxygen species, possibly leading to permanent cell damage [12].

Aside from the possible direct effects of (molecular) (CE)US imaging and the use of contrast agents, there are other, more general aspects of imaging procedures, which have to be considered. For example, rodents, in contrast to humans, need to be immobilized with an anesthetic to obtain accurate results during the whole imaging process. Isoflurane anesthesia, which is often used for this purpose, is known to modify and interact with many receptors such as the NMDA receptor or the acetylcholine receptor 1 [13–17]. These widespread sites of action are discussed to be the cause of various pathophysiological and cognitive alterations, for example, temporary and sustained impairments in learning/memory or intensified anxiety-related behavior [18–22]. The risk of such adverse side effects increases with the length and repetition of anesthesia, which is unavoidable when performing longitudinal studies [21, 23]. Additionally, the necessity to remove fur at the site of US examination can lead to behavioral alterations in rodents, as the fur, apart from thermoregulatory functions, is also used for signaling, communication, or defense [24, 25]. Moreover, the correct needle placement into the tail vein of mice, during the injection of US contrast agents, is a crucial step. It

needs to be performed by a well-trained person to prevent severe bruising or scarring, which can lead to difficulties with subsequent injections. Those injuries can also induce inflammation at the injection sites and therefore, influence study results such as blood parameters [26].

In conclusion, all these conditions may have a negative impact on animal welfare and thus significantly affect the overall course and results of experiments. Despite the increasingly widespread use of noninvasive imaging in preclinical settings, no systematic study has been performed to assess the potential impact of US on animal well-being. Therefore, we investigated the possible effects of repeated US and CEUS on the welfare of healthy mice. Moreover, we evaluated the effects of CEUS and molecular CEUS protocols on the well-being and study outcomes in regorafenib-treated and untreated tumor-bearing mice.

Materials and Methods

Animal Experiments

All animal experiments were approved by the German State Office for Nature, Environment, and Consumer protection (LANUV) North Rhine-Westphalia. One hundred forty female Balb/cAnNRj mice (Janvier Labs, Saint Berthevin, France), aged 10–12 weeks, were housed (maximum of 5 mice/cage) according to the guidelines of the "Federation for Laboratory Animal Science Associations" (www.felasa.eu) on spruce granulate bedding. These guidelines include a temperature- and humidity-controlled environment (20°C–24°C; 45%–65%) with a 12-h light/dark cycle. Food (Sniff GmbH, Soest, Germany) and acidified water were available ad libitum. One nestlet per cage was provided to enable nest building. All efforts were made to minimize the number of animals used in this study and their suffering.

Animal Welfare Assessment

We monitored animal welfare by using a daily score sheet and acquiring Rotarod performance, heart rate, and by the assessment of fecal corticosterone metabolites (FCMs) twice a week (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000524431 for all online suppl. material). All mice underwent a training phase prior to the first US imaging for acclimation to the behavioral tests, enabling the determination of accurate baseline values for the analyzed criteria. Welfare monitoring and behavioral tests were consistently carried out during the morning to avoid possible diurnal effects. Before euthanasia, a final assessment of all parameters was performed on the last day of the experiment. In addition, retrobulbar blood was obtained for hemogram analysis.

Welfare Monitoring (Score Sheet)

We used a standardized score sheet based on the general guidelines for assessing pain, stress, and discomfort in laboratory animals [27]. It was adapted to the specific conditions (tumor growth, antitumor treatment) in our study (online suppl. Table S1). Possible alterations in body weight, general state (e.g., fur appearance), clinical symptoms (e.g., heart rate), spontaneous behavior, and treatment-specific parameters (e.g., tumor growth, antitumor treatment) were evaluated according to a point grading system. No changes in the physiological state were graded with 0 points, whereas 20 points or higher marked the highest severity and was stated as a humane endpoint. Animals reaching this endpoint were taken out of the study.

Rotarod Performance

The Rotarod test (Panlab Harvard Apparatus, Barcelona, Spain) was used to assess possible changes in motor coordination and balance of rodents. For this purpose, mice were placed on a spinning cylinder with a start velocity of 4 rotations per minute (rpm), steadily increasing to a maximum speed of 40 rpm within 5 min. The time and speed at which the rodents fell from the rotating cylinder were recorded. Each measurement was consecutively repeated twice. The baseline values were set to 100% and the percentage change of the following time points was calculated individually.

Heart Rate Assessment

To assess changes in the animals' heart rate, conscious mice were carefully restrained in a plexiglass holder. The tail of the rodent was secured in an occlusion and volume pressure cuff, to accurately determine the heart rate by volume pressure recording using the CODA System (Kent Scientific Corporation, Torrington, CT, USA). A tempered panel guaranteed a stable body temperature. Each measurement consisted of 15 repetitions and was analyzed by the supplier's software Coda 4.1 (Kent Scientific Corporation). The baseline values were set to 100% and the percentage change of the following time points was calculated individually.

Measurement of FCMs

Fecal samples of each mouse were collected during the Rotarod tests and heart rate measurements. Dried samples (50 mg) were dissolved in 80% methanol (Merck, Darmstadt, Germany) overnight at 4°C and then homogenized and centrifuged (10 min; 3,000 g relative centrifugal acceleration, Fresco 21 & Pico 21 Heraeus, Hanau, Germany) at the next day. The samples were analyzed with a 5α-pregnane-3β, 11β, 21-triol-20-one enzyme immunoassay to assess FCM concentrations [28, 29].

Hemograms

Retrobulbar sinus puncture was performed to obtain blood samples from the anesthetized animals on the last day of experiments. Subsequently, various blood values such as leukocyte, erythrocyte, and thrombocyte counts, as well as hemoglobin and hematocrit concentrations were determined using a blood screening device (Celltac alpha MEK-6550, Nihon Kohden, Shinjuku, Japan). In addition, serum samples were prepared and analyzed using a Rodent Stress Hormone Magnetic Bead Panel Kit (Milliplex MAP Panel, Merck, Darmstadt, Germany) and measured with a Luminex MAGPIX system (Thermo Fischer, Waltham, MA, USA) to detect the amount of corticosterone in the blood serum.

Repeated US and CEUS Examinations in Healthy Mice

First, we assessed the influence of repeated US and CEUS imaging on healthy mice. Therefore, 40 female Balb/c mice were randomized to the following groups: (i) control; (ii) isoflurane; (iii) US; (iv) CEUS. In line with the concept of 3R, data of healthy mice of control and isoflurane groups that were investigated at identical

time points and by the same researchers were reused from a previous study published by Baier et al. [30]. All animals underwent severity assessment tests as described above (CE)US imaging was applied on the right mammary fat pad three times a week for 4 weeks. Mice of the isoflurane group (ii) received anesthesia with 2% isoflurane in oxygen for 30 min on the same days when (CE) US measurements were performed (online suppl. Fig. 1a).

Repeated CEUS and Molecular CEUS Examinations in Tumor-Bearing Mice

CEUS and molecular CEUS measurements were performed on 4T1 tumor-bearing mice to assess the possible impact of these diagnostic imaging methods on animal well-being and on the response to regorafenib treatment. Therefore, anesthetized mice (n = 94) received an injection of 4×10^4 murine triple-negative breast cancer cells (4T1; ATCC CRL-2539, Manassas, VA, USA) in 50 µL RPMI 1640 cell culture medium into the right mammary fat pad. Tumor growth was measured daily with a Vernier caliper. The mice received a daily oral dose of the multikinase inhibitor regorafenib (10 mg/kg body weight; dissolved in polyethylene glycol 400, 1,2-propandiol, pluronic F68; Merck, Darmstadt Germany) or the equivalent amount of vehicle solution, starting from day 6. The rodents were randomized to the following experimental groups: (i) control vehicle (n = 12) or regorafenib (n = 10); (ii) isoflurane vehicle (n = 14)/regorafenib (n = 10); (iii) CEUS vehicle (n = 12)/regorafenib (n = 10); (iv) molecular CEUS vehicle (n = 14)/regorafenib (n = 12). CEUS and molecular CEUS measurements under isoflurane anesthesia or isoflurane anesthesia alone (30 min, 2% isoflurane in O_2) were performed three times within 2 weeks (online suppl. Fig. 1b).

US, CEUS, Molecular CEUS Protocol

(Molecular) (CE)US measurements were performed under isoflurane anesthesia (2% in oxygen) using the Vevo2100 small animal US scanner (VisualSonics, Toronto, ON, Canada) equipped with the MS250 transducer. The transducer was placed on the mammary fat pad (healthy mice) or the tumor and connected to the skin using US gel (ArneMaas, Borken, Germany). First, a 3D measurement of the whole fat pad containing the tumor was performed in contrast mode (18 MHz, 4% power, 0.12 MPa peak negative pressure, MI 0.04) without microbubbles. For CEUS, 5×10^7 microbubbles/50 µL 0.9% NaCl non-targeted phospholipid microbubbles (VevoMicromarker®, VisualSonics) were injected intravenously into a lateral tail vein and their inflow was recorded in one plane for 60 s (framerate 10 fps). Immediately afterward, a second 3D measurement was carried out to assess the microbubble signals within the entire fat pad and tumor.

The molecular CEUS imaging followed the same protocol as described above. In contrast to CEUS, phospholipid microbubbles (target-ready VevoMicromarker®, VisualSonics) coated with an anti-VEGFR2 antibody (eBiosciences, San Diego, CA, USA), were injected into a lateral tail vein (5×10^7 microbubbles/50 µL 0.9% NaCl). After the first series of measurements (3D baseline measurement, recording the inflow of microbubbles, second 3D measurement), a period of 8 min allowed the microbubbles to adhere to the specific receptor. Thereafter, a 3D measurement was performed to assess the signal of bound microbubbles in the whole fat pad, including the tumor. Then, a destruction-replenishment measurement was performed in one slice, which included a destructive US pulse (18 MHz, 100% power, 3 MPa peak negative pressure, MI 1.6) to detect bound microbubbles. Subsequently, a destructive 3D sequence was applied to destroy all microbubbles in the entire fat pad, including the tumor, followed by a final 3D measurement. During the whole time, the mice were placed on a tempered platform to maintain their body temperature and, if necessary, the fur above the imaged fat pad or tumor was removed with hair removal cream (Veet, Slough, UK). In order to avoid skin irritations, the incubation time of the hair removal cream was limited to a maximum of approximately 15 s. Intravenous injections were performed by an experienced researcher to reduce the risk of injuries at the injection site. During the daily welfare assessment, the tail was examined for signs of inflammation or pain.

Organ and Tumor Tissue Preparation

On the last day of the experiment, healthy mice were euthanized by cervical dislocation. Tumor-bearing mice were first anesthetized by an intraperitoneal injection of ketamine/xylazine (120 mg/kg bodyweight ketamine and 16 mg/kg bodyweight xylazine in 0.9% NaCl; 30 µL/10 g body weight) and euthanized by heart perfusion with 10-mL phosphate buffer saline (Merck, Darmstadt, Germany) through the left ventricle. The main organs (brain, heart, lungs, liver, spleen, and kidneys) and tumors were removed and examined for abnormalities in weight and appearance.

Statistical Analysis

For statistical analyses the data were tested for normality and analyzed with a one-way analysis of variance, followed by a Dunnett post hoc test on a 95% confidence interval (SPSS, IBM Corp, v25, Sanborn, NY, USA, academic license; GraphPad Prism 5, v5.01, San Diego, CA, USA, academic license). A probability (p) value of less than 0.05 was considered to be statistically significant. All results are reported as means ± standard deviations.

Results

Influence of Repeated US Examinations on Healthy Mice

In the first part of this study, the possible influence of repeated US measurements on the well-being of healthy Balb/c mice was investigated. The daily welfare monitoring with our score sheet (online suppl. Table S1) showed a mild burden after US imaging, but none of the examined mice exceeded a total score of 5 points (Fig. 1a; online suppl. Table S2). The same score was found for animals of the control and isoflurane anesthesia groups. Bodyweight and heart rate remained stable throughout the observation period in US-examined mice and were comparable to the control animals (p = 0.77, p = 0.53; online suppl. Fig. 1a; Fig 1b). Furthermore, Rotarod performance was not affected by US (Fig. 1c).

Corticosterone (metabolite) concentrations in blood or feces were not altered by US imaging and comparable to control animals at the end of the experiment (p = 0.91, p = 0.98; Fig. 2a–b). In line with these results, US did not

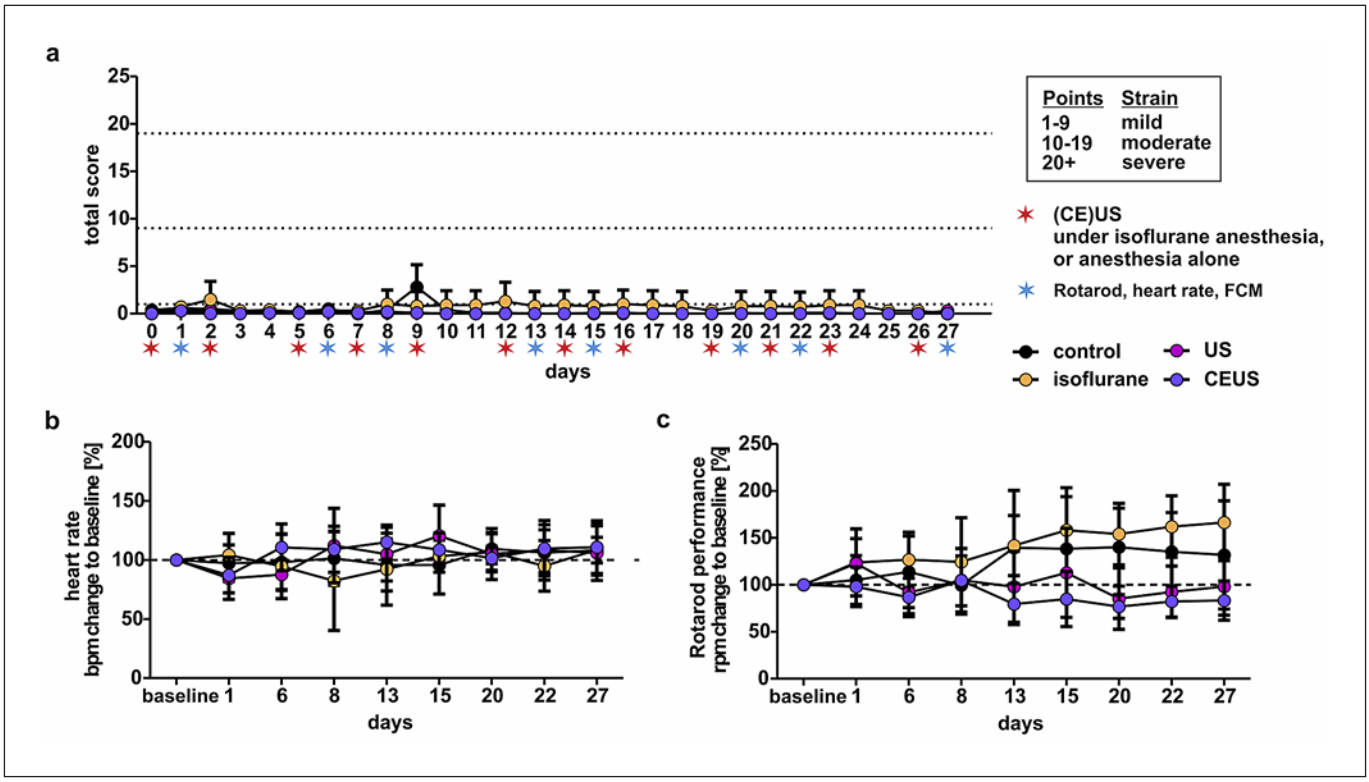

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Fig. 1. Influence of repeated (CE)US imaging on animal welfare of healthy Balb/c mice. **a** Score sheet assessment (body weight, general condition, spontaneous behavior, clinical symptoms) indicates a mild burden (1–9 points) for all examined mice, regardless of (CE)US imaging. **b** Heart rate remains stable throughout the research period. **c** Longitudinal Rotarod performance (rotations per minute) is not affected by repeated (CE)US scans. Results are presented as means ± standard deviations. BPM, beats per minute; RPM, rotations per minute; (CE)US, (contrast-enhanced) ultrasound.

affect blood parameters such as leukocyte-, erythrocyte-, thrombocyte counts as well as hemoglobin and hematocrit values ($p = 0.86$, $p = 0.94$, $p > 0.99$, $p = 0.61$, $p = 0.31$; Fig. 2c; online suppl. Fig. 2b, c; online suppl. Table S3). Five animals of the control and isoflurane group had to be excluded from hemogram analysis due to an internal error of the measurement device. The spleen weight of US-examined mice was slightly reduced in comparison to control animals ($p = 0.12$; Fig. 1d). The same effect was also visible for mice that received only isoflurane anesthesia ($p < 0.05$) so that the effect is rather related to the anesthetic. The weights of all other organs (brain, heart, lungs, liver, and kidneys) were comparable among the groups (online suppl. Table S4).

Influence of Repeated CEUS Examinations on Healthy Mice

In another group of mice, we investigated if repeated CEUS, which uses gas-filled microbubbles to visualize the vasculature, has an effect on well-being of healthy Balb/c mice. Score sheet evaluations showed a mild burden (maximum 5 points) for animals examined by CEUS, which was also observed for the control and US groups (Fig. 1a). Other longitudinal severity parameters such as bodyweight, heart rate, and Rotarod performance remained unchanged after repeated CEUS imaging and were comparable to non-imaging groups ($p = 0.76$, $p = 0.14$, $p = 0.1$; online suppl. Fig. 2a; Fig. 1b, c).

In line with results from US-treated mice, CEUS did not affect the concentrations of corticosterone (metabolites) in serum and feces ($p = 0.59$, $p = 0.99$; Fig. 2a, b; online suppl. Table S2). Furthermore, hemogram analyses were also comparable to animals of the control, isoflurane, and US groups (Fig. 2c; online suppl. Fig. 2b, c; online suppl. Table S3). Spleen weights were slightly lower in CEUS-examined mice, as also observed for mice of the isoflurane and US groups ($p = 0.82$; Fig. 2d). In addition, there were no alterations in organ weight (brain, heart, lungs, liver, and kidneys) after repeated CEUS (online suppl. Table S4).

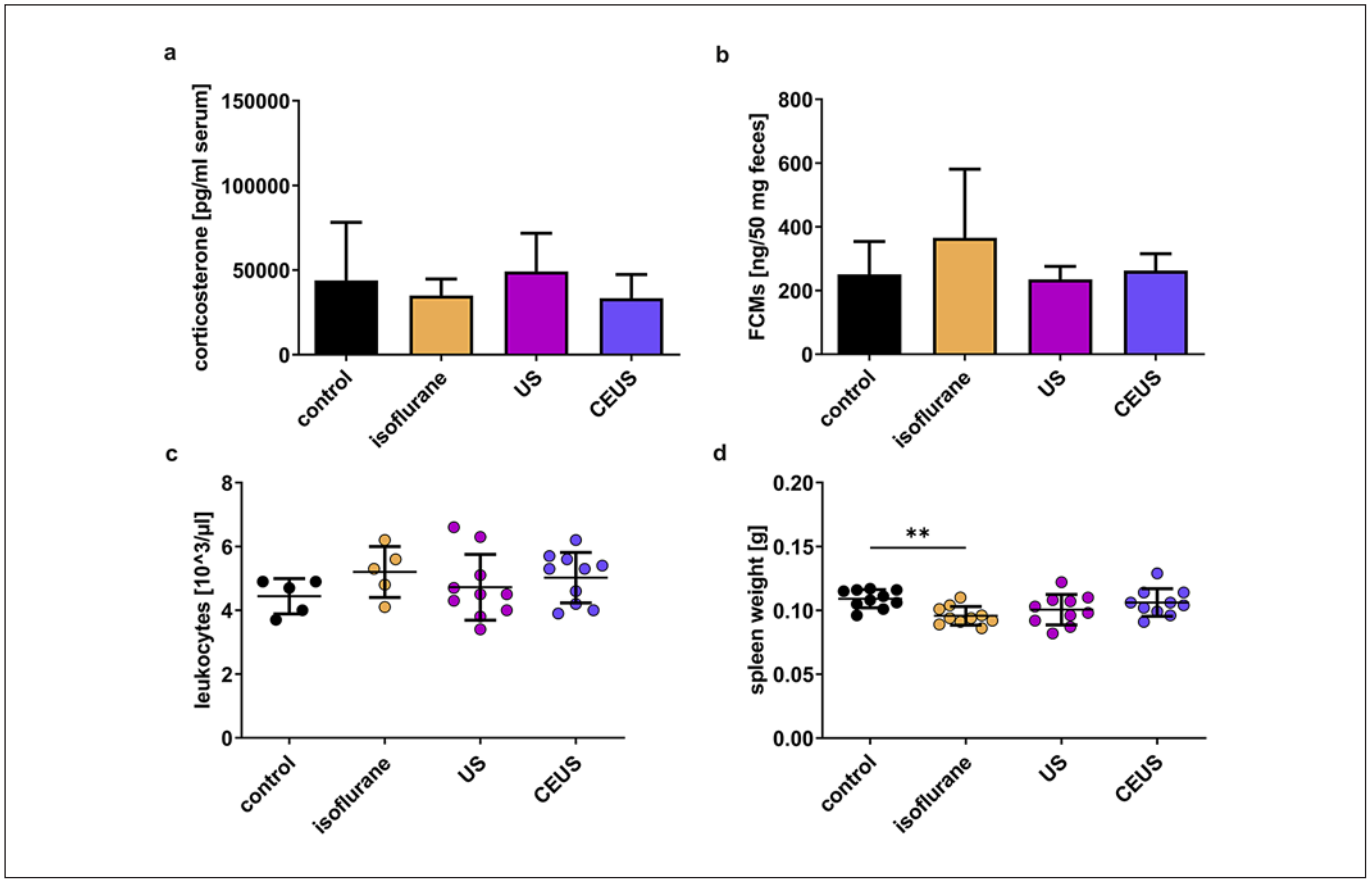

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Fig. 2. Influence of repeated (CE)US imaging on corticosterone metabolite concentrations in feces and blood serum, leukocytes, and spleen weights of healthy Balb/c mice. **a** Corticosterone levels in blood serum are not affected by repeated (CE)US examinations. **b** Levels of FCMs do not change over time, regardless of (CE)US imaging. **c** Leukocyte counts are not altered by (CE)US scans. **d** Spleen weights are reduced due to isoflurane anesthesia ($p < 0.01$), but not further affected by (CE)US imaging. **b–d** Data were collected on day 27 of the experiment. Results are presented as means ± standard deviations. (CE)US, (contrast-enhanced) ultrasound. $^{**}p < 0.01$.

Influence of Tumor Growth and Regorafenib Treatment on Tumor-Bearing Mice

In the second part of the study, we wanted to investigate the influence of diagnostic (molecular) CEUS techniques on 4T1 breast cancer-bearing mice. Score sheet evaluations displayed gradually increased scores for all tumor-bearing mice (Fig. 3a). However, maximum scores did not exceed the mild burden range (1–9 points) and are strongly connected to tumor growth. Furthermore, all tumor-bearing mice had significantly higher leukocyte counts (especially neutrophils) and spleen weights, compared to healthy mice ($p < 0.01$, $p < 0.01$; Fig. 4c, d; online suppl. Fig. 3b). Tumor weights were significantly lower in regorafenib-treated mice in comparison to vehicle-treated control ($p < 0.05$), while there was no difference detectable within the regorafenib-treated groups. Regorafenib treatment resulted in elevated corticosterone (metabolite) concentrations in both, blood and feces when compared to vehicle-treated mice ($p = 0.63$, $p < 0.05$; Fig. 4a, b; online suppl. Table S5). However, other severity parameters (score sheets, body weight, heart rate, and Rotarod performance) and pathological parameters (hemogram analysis, organ weights) were comparable between all regorafenib- and vehicle-treated mice (online suppl. Fig. 3a; Fig. 3a–c; online suppl. Table S5).

Influence of Repeated CEUS on 4T1 Tumor-Bearing Mice

In the first cohort, we investigated the effects of CEUS, which can be used to assess tumor vascularization, on the animals' well-being and therapy response to regorafenib. Score sheet evaluations, body weight, heart rate, and Rotarod performance were not altered by CEUS imaging

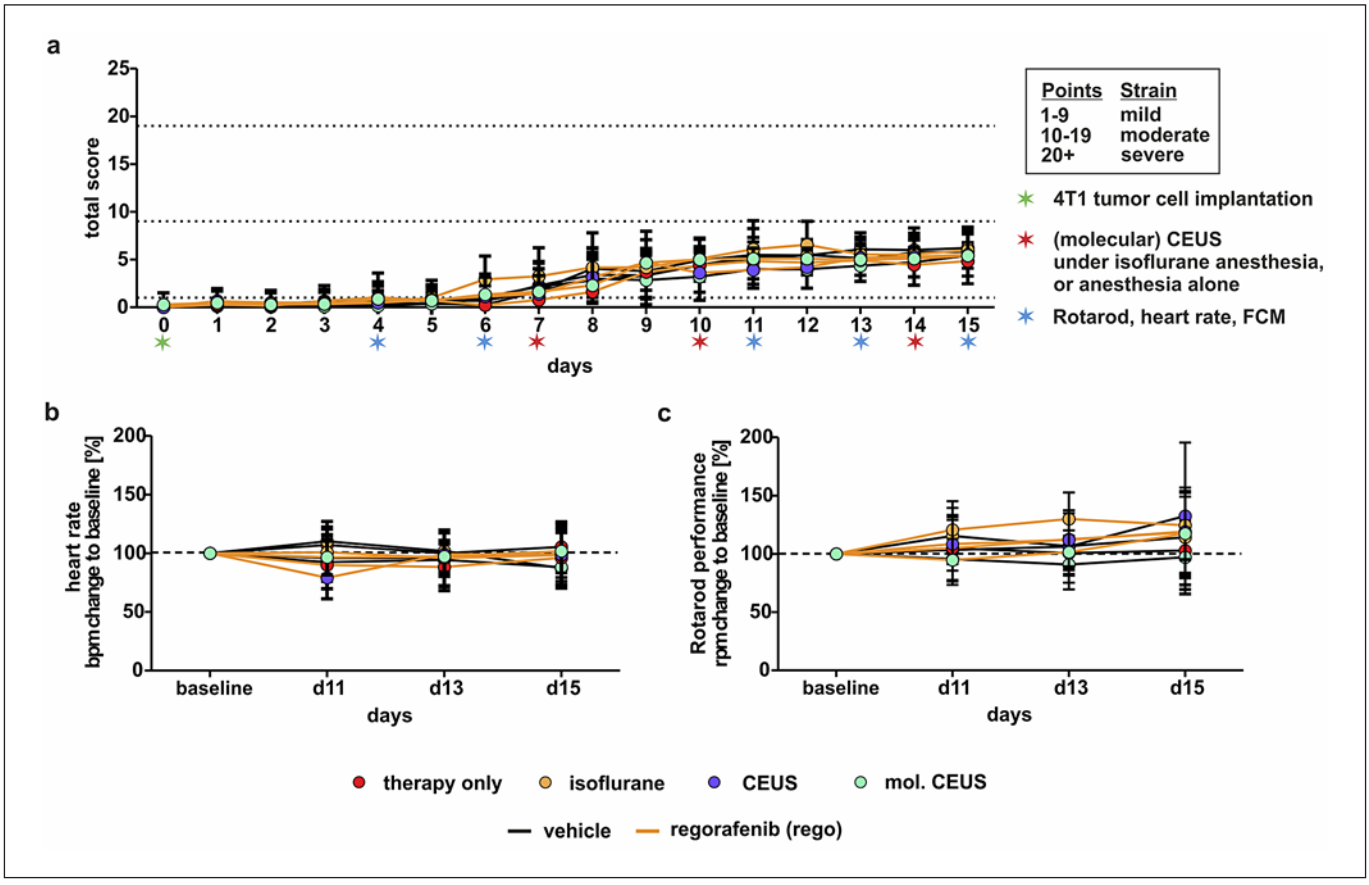

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Fig. 3. Influence of repeated CEUS and molecular CEUS imaging on the welfare of 4T1 tumor-bearing Balb/c mice. **a** Score sheet assessment (body weight, general condition, spontaneous behavior, clinical symptoms, body conditioning score, orthotopic tumor growth) indicates a mild burden (1–9 points) for all mice due to tumor growth, regardless of CEUS, molecular CEUS, or regorafenib treatment. **b** Heart rates are not altered throughout the study. **c** Longitudinal Rotarod performances (rotations per minute) are not affected by repeated CEUS scans or regorafenib treatment. Results are shown as means ± standard deviations. BPM, beats per minute; RPM, rotations per minute; CEUS, contrast-enhanced ultrasound; mol., molecular.

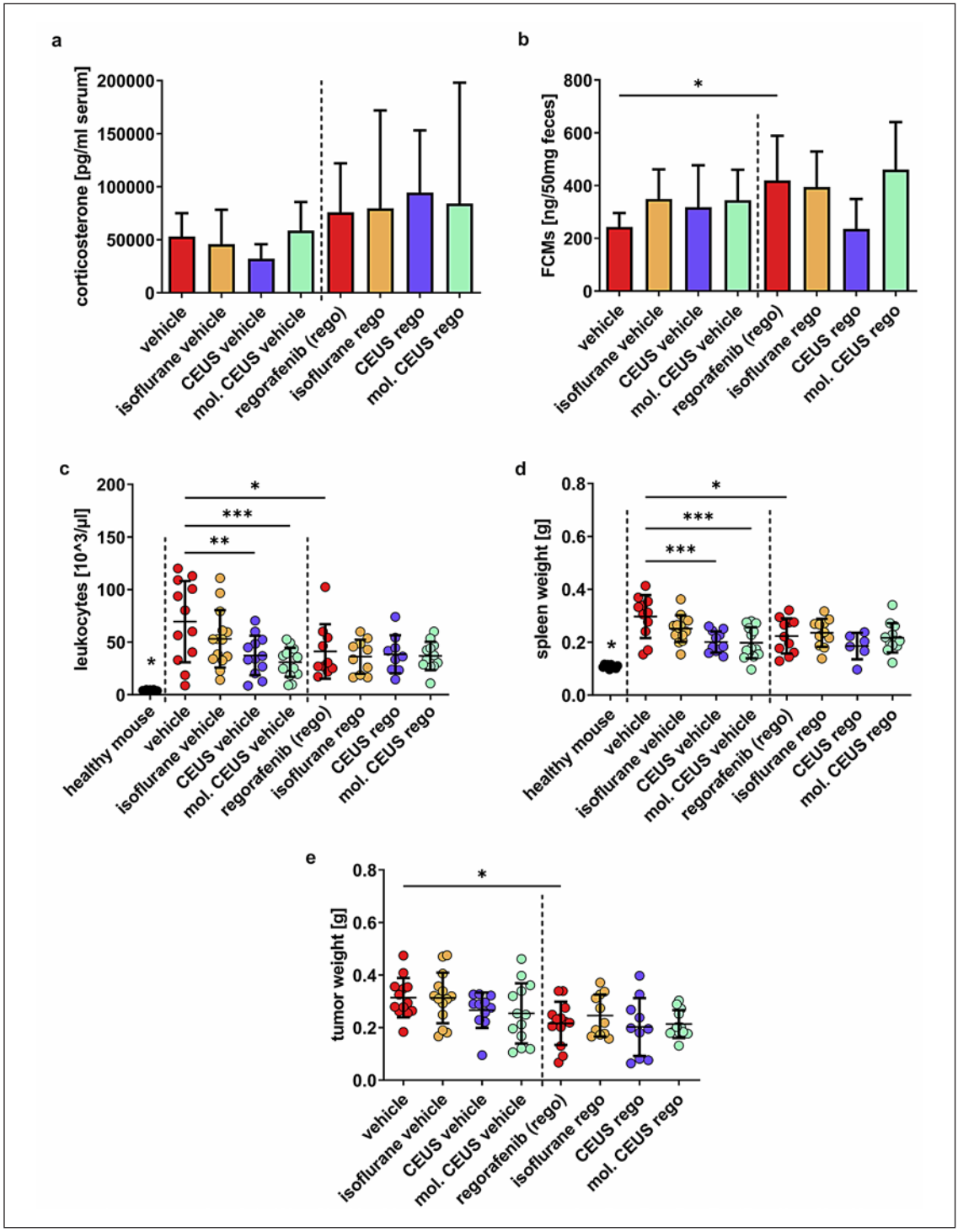
($p = 0.29$, $p = 0.32$, $p = 0.22$; online suppl. Fig. 3a; Fig. 3a–c; online suppl. Table S5). Assessment of corticosterone (metabolites) in serum and feces were comparable in all vehicle-treated animals ($p = 0.94$, $p = 0.61$; Fig. 4a, b; online suppl. Table S5). Interestingly, leukocyte counts were lower after repeated CEUS compared to the vehicle-treated control and isoflurane groups ($p = 0.005$, $p = 0.35$; Fig. 4c). All other blood parameters were not affected by CEUS (online suppl. Fig. 3c, d; online suppl. Table S6). In line with leukocyte counts, the spleen weights were significantly lower after CEUS in vehicle-treated mice compared to vehicle-treated animals of the control group ($p < 0.001$; Fig. 4d). Spleen weights after repeated CEUS were lower than spleen weights in the isoflurane vehicle group ($p = 0.12$). Furthermore, the tumor weights were slightly lower after CEUS compared to those in the vehicle-treated control and isoflurane groups ($p = 0.28$; Fig. 4e; online suppl. Table S7). All other organ weights (brain, heart, lungs, liver, and kidneys) were comparable (online suppl. Table S7). Within regorafenib-treated animals, all welfare parameters, hemogram analyses, and organ weights were comparable between the control, isoflurane, and CEUS groups (Fig. 3, 4; online suppl. Fig. 3; online suppl. Tables S5–7).

Influence of Repeated Molecular CEUS on 4T1 Tumor-Bearing Mice

In the second cohort of mice, we applied molecular CEUS to assess the VEGFR2 expression in angiogenic tumor vessels. The application of VEGFR2-targeted microbubbles and the destruction-replenishment technique, that is used to assess the microbubble signal, can lead to

a short, but stronger interaction with the endothelium, compared to CEUS and could, therefore, possibly result in different effects on the tumor.

In line with tumor-bearing mice of the control, isoflurane, and CEUS groups, molecular CEUS did not affect score sheet evaluations, bodyweight, heart rate, and Rotarod performance of vehicle-treated mice compared to the control and isoflurane groups ($p = 0.93$, $p = 0.13$, $p = 0.26$; online suppl. Fig. 3a; Fig. 3a–c; online suppl. Table S5). These findings could be confirmed by comparable



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Eur Surg Res 2023;64:77–88
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corticosterone (metabolite) concentrations in serum and feces samples ($p = 0.99$, $p = 0.37$; Fig. 4a, b). After CEUS, significantly less leukocytes were counted in vehicle-treated animals examined with molecular CEUS compared to the control group ($p < 0.001$; Fig. 4c). Moreover, leukocyte counts after molecular CEUS were lower than in the isoflurane vehicle group ($p = 0.06$; Fig. 4c). No changes in numbers of erythrocytes, thrombocytes as well as in hemoglobin and hematocrit values after repeated molecular CEUS were observed ($p = 0.83$, $p = 0.84$, $p = 0.96$, $p = 0.85$; online suppl. Fig. 3c, d; online suppl. Table S6). Again, as for the CEUS group, spleen weights were significantly lower in vehicle-treated animals after molecular CEUS, compared to controls without US ($p < 0.001$; Fig. 4d). Spleen weights after repeated molecular CEUS were lower than spleen weights of isoflurane vehicle group ($p = 0.08$). Molecular CEUS did not affect the weight of other organs (brain, heart, lungs, liver, and kidneys) (online suppl. Table S7). In line with the results after CEUS, tumor weights of vehicle-treated animals were slightly lower after molecular CEUS compared to the control and isoflurane groups ($p = 0.20$; Fig. 4e). Furthermore, within regorafenib-treated animals, no differences in welfare parameters, corticosterone metabolite concentrations, hemograms, or organ weights were detected after molecular CEUS compared to the control, isoflurane, and CEUS groups (Fig. 3, 4; online suppl. Fig. 3; online suppl. Tables S5–7).

Fig. 4. Influence of repeated CEUS and molecular CEUS imaging on corticosterone metabolite concentrations in feces and blood serum, leukocyte counts, spleen, and tumor weights of 4T1 tumor-bearing Balb/c mice. **a** Corticosterone levels in blood serum are not affected by repeated CEUS examinations but increased in all regorafenib-treated mice. **b** Levels of FCMs are increased in all regorafenib-treated mice over time (except for CEUS), regardless of molecular CEUS imaging. **c** Leukocyte counts are increased in all groups of tumor-bearing mice in comparison to healthy mice ($p < 0.05$). Compared to the vehicle-treated group, significantly less leukocytes are counted in animals examined with CEUS ($p < 0.01$) and molecular CEUS ($p < 0.001$). **d** Spleen weights are significantly lower after CEUS and molecular CEUS in vehicle-treated animals, compared to vehicle-treated controls ($p < 0.001$, $p < 0.001$). **e** Tumor weights are significantly lower in regorafenib-treated mice in comparison to vehicle-treated controls ($p < 0.05$). This effect is not clearly visible in animals scanned with (molecular) CEUS (data were collected on day 15 of the experiment). CEUS, contrast-enhanced ultrasound; mol., molecular. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion/Conclusion

According to the EU Directive 2010/63/EU Annex VIII, noninvasive imaging of animals is considered as of mild severity [31]. However, there is no scientific reasoning on how this severity classification was achieved, and there are often several factors (e.g., imaging, anesthesia, administration of a therapeutic agent, tumor growths) that have to be considered to estimate the total impact of an experiment on the animals' welfare. Although US is clinically well-established and considered harmless in patients, therapeutic US can also be used to ablate tumors [32] or manipulate the immune cell infiltration into tumors [33]. In addition, microvascular damage has been reported in rats even after diagnostic CEUS [9, 10]. Therefore, we here present a systematic evaluation of the influence of different preclinical US imaging methods on the welfare of healthy and tumor-bearing mice and on the outcome of regorafenib antitumor therapy.

We could show that healthy mice displayed only a mild burden at the end of the experiments and that the well-being of animals was not altered after repeated isoflurane anesthesia, US, or CEUS. These results confirm the severity classification for noninvasive imaging of the EU Directive 2010/63/EU Annex VIII.

However, we observed a reduction in spleen weight in all groups of healthy mice which received isoflurane anesthesia. Although this physiological alteration has not been described for mice yet, other anesthetic drugs such as acepromazine, propofol, or xylazine were reported to have a similar effect on canine splenic weight, probably caused by an increase in vagal tone or sympatholytic effects [34–36]. Therefore, the possible effects of anesthesia, which is necessary for imaging procedures, must be considered.

In the second part of the study, we evaluated the impact of CEUS and molecular CEUS on tumor-bearing mice. Here, additionally to (molecular) CEUS imaging, tumor growth and the application of the antitumor drug regorafenib can impact animals' welfare. In this regard, the EU Directive 2010/63/EU Annex VIII classifies tumor growth and application of sublethal doses of chemotherapeutics as of moderate severity [31]. Our score sheet evaluations resulted in a mild burden (1–9 points) at the end of the study for all tumor-bearing animals. No alterations after CEUS, molecular CEUS, or regorafenib treatment could be observed. However, we detected a gradual increase in score points that could be attributed to the continuous growth of the tumors. It needs to be mentioned that a long-lasting mild burden of animals is also classi-

fied as moderate severity [31]. Therefore, we can also confirm the moderate severity classification for our protocol of noninvasive (molecular) CEUS imaging in tumor-bearing mice receiving regorafenib chemotherapy as described in the EU directive.

Despite the mild burden that was detected by welfare assessment, all tumor-bearing mice displayed leukocytosis and splenomegaly, which can be associated with tumor growth [37, 38]. In detail, neutrophils were the most strongly increased leukocyte fraction. Leukocytes are the first line of defense of the innate immune system and particularly elevated during cancer development [39]. Isoflurane anesthesia reduced the tumor-related leukocytosis and splenomegaly, which can be explained by the inhibition of cellular immune responses that were reported in humans after isoflurane anesthesia [40]. Moreover, reduced leukocyte counts after isoflurane anesthesia were also observed in various animal studies [41–43]. Interestingly, the normalization of leukocyte counts and spleen weights was more pronounced in tumor-bearing animals examined with CEUS or molecular CEUS compared to isoflurane anesthesia alone. Several studies observed phagocytosis of microbubbles by activated cells [44–47], which may have influenced their biological behavior (e.g., their homing and evasion from bone marrow and spleen). Moreover, Lindner et al. [44] showed that phospholipid- and albumin-coated microbubbles, which were phagocytosed by activated neutrophils and monocytes, remain acoustically active and responsive to US in vitro, which might have affected survival and triggered a response of the immune cell fraction in the insonnated tumors. In our study, we used Vevo micromarker and target-ready Vevo micromarker microbubbles (shell: phospholipid; core: C_4F_{10}/N_2; diameter: 1.3 μm) which are quite similar in their constitution to the microbubbles (shell: phospholipid; core: C_4F_{10}; diameter: 2.8–4.1 μm), which were used by Lindner et al. [44]. Furthermore, the microbubbles used in our study are even smaller in diameter, which is also notable as smaller probe size is known of being a critical uptake-determining parameter of phagocytizing cells [48]. However, further research is needed to investigate if the reduced leukocyte counts and spleen weights solely occur when using the preclinical microbubbles and the imaging protocol of the present study, or if these effects also occur with clinically approved contrast agents and clinical (molecular) CEUS settings. Furthermore, no significant differences in the investigated welfare and physiological parameters could be detected between animals examined with CEUS or molecular CEUS, although additional destructive pulses (MI 1.6) were applied on the tumor during molecular CEUS. However, the MI of these destructive pulses is below the clinically relevant safety limit of MI 1.9 [5], and they are only applied once at the tumor, therefore, the onetime destruction of microbubbles in the tumor might not be sufficient to create significantly greater bioeffects. Despite systemic changes in the immune system, tumor weights of vehicle-treated animals were just slightly lower but not significantly altered after CEUS and molecular CEUS compared to the control groups. In this context, potential pathophysiological and immunological effects in tumors, such as immune cell infiltration, need to be analyzed in more detail to unravel the impact of CEUS and molecular CEUS on tumor biology.

In order to investigate the possible impact of CEUS and molecular CEUS imaging on antitumor therapy response, mice were treated with the chemotherapeutic agent regorafenib. This multikinase inhibitor is known to inhibit VEGFR 1–3, TIE2, KIT, RET, RAF-1, BRAF, BRAFV600E, PDGFR, FGFR, and therefore, has both antitumoral and antiangiogenic properties [49, 50], which has the benefit that a possible influence on both mechanisms of action could be detected. Furthermore, by using phospholipid microbubbles coated with an anti-VEGFR2 antibody we wanted to investigate possible direct interactions and interferences of the contrast agent with regorafenib, which is also affecting VEGFR2-signaling. Regorafenib treatment resulted in elevated values of serum or fecal corticosterone (metabolites) in all treated animals. The simultaneous increase in corticosterone concentrations that went along with decreased thyroid hormone levels in the plasma of regorafenib-treated patients, could explain the observed changes in the endocrine system [51, 52]. Furthermore, regorafenib induces myelosuppression by inhibiting tyrosine kinases [53]. Therefore, leukocytosis and splenomegaly were less pronounced in regorafenib-treated animals and comparable within all regorafenib-treated groups. This myelosuppressive effect of regorafenib might be stronger than the effects of isoflurane and (molecular) CEUS and therefore only the effect of regorafenib is detectable. Despite these general findings resulting from regorafenib therapy, we could not detect an influence of CEUS and molecular CEUS on regorafenib-mediated tumor growth suppression.

It is important to note that our preclinical imaging protocol influenced the study outcome. In this regard, the comparison between animals of the control groups (without imaging) revealed a significant reduction in leukocyte counts, spleen weights, and tumor weights after regorafenib treatment. In contrast, no significant differences were found when comparing vehicle-treated and rego-

rafenib-treated animals of the same imaging group (e.g., CEUS vehicle vs. CEUS rego). These results highlight the need to include appropriate control groups to unravel an experimental bias that could also influence the reproducibility of experiments.

In conclusion, we show that none of the applied (molecular) (CE)US protocols had a detectable influence on the welfare of healthy and 4T1 tumor-bearing mice. Neither the increased number of (CE)US examinations, as performed in healthy individuals, nor the application of high-pressure pulses during molecular CEUS in tumor-bearing mice, had an effect on animals' well-being. Therefore, we can confirm that the severity classification, as reported by Annex VIII of the EU Directive 2010/63/EU, to induce a mild burden in healthy and tumor-bearing animals is justifiable for the US, CEUS, and molecular CEUS protocols used in our study. However, alterations in hemogram analyses as well as in spleen weights indicate that isoflurane anesthesia, CEUS, and molecular CEUS might alter a tumor-related immune reaction and resulted in a different interpretation of study outcomes. These findings are particularly important to avoid unwanted experimental bias in immunotherapy-related research and potentially open new immunotherapeutic perspectives. Therefore, further experiments using different types of microbubbles and (molecular) (CE)US settings are needed to unravel the influence on tumor growth and therapeutic applications.

Statement of Ethics

The study protocol, including all animal experiments, was reviewed and approved by the German State Office for Nature, Environment, and Consumer protection (LANUV) North Rhine-Westphalia, approval number 84-02.04.2017.A328.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Fabian Kiessling and Anne Rix designed the study and Anne Rix and Jasmin Baier carried out the experiments. Renée M. Girbig performed serum corticosterone analysis, data preparation, and literature research and wrote the research article. Hemogram analyses were done by the Institute for Laboratory Animal Science and Experimental Surgery, Medical Faculty, RWTH Aachen (Rene Tolba). Fecal corticosterone metabolite analyses were performed by Rupert Palme, Department of Biomedical Sciences, University of Veterinary Medicine Vienna. Anne Rix and Fabian Kiessling provided supervisory support and edited the article. All authors edited, revised, and finally approved the published version of the manuscript. They agree that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

All data are stored on servers of the RWTH Aachen University considering the FAIR (findable, accessible, interoperable, reusable) criteria and can be provided to other scientists on request.

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