Comparison of photoacoustic and fluorescence tomography for the \textit{in vivo} imaging of ICG-labelled liposomes in the medullary cavity in mice

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\textbf{ABSTRACT}

Few reports quantitatively compare the performance of photoacoustic tomography (PAT) versus fluorescence molecular tomography (FMT) \textit{in vivo}. We compared both modalities for the detection of signals from injected ICG liposomes in the tibial medullary space of 10 BALB/c mice \textit{in vivo} and \textit{ex vivo}. Signals significantly correlated between modalities ($R^2 = 0.69$) and within each modality \textit{in vivo} versus \textit{ex vivo} (PAT: $R^2 = 0.70$, FMT: $R^2 = 0.76$). Phantom studies showed that signals at 4 mm depth are detected down to 3.3 ng ICG by PAT and 33 ng by FMT, with a nominal spatial resolution below 0.5 mm in PAT and limited to 1 mm in FMT. Our study demonstrates comparable \textit{in vivo} sensitivity, but superior \textit{ex vivo} sensitivity and \textit{in vivo} resolution for our ICG liposomes of the VevoLAZR versus the FMT2500. PAT provides a useful new tool for the high-resolution imaging of bone marrow signals, for example for monitoring drug delivery.

1. Introduction

Bone imaging is needed both in the preclinical and clinical setting, mainly for bone-related diseases like osteoporosis, osteoarthritis, bone metastases or diseases affecting the bone marrow. In the preclinical field, bone imaging additionally is a common tool for basic research questions. Therefore, options to non-invasively image signals from the intact medullary space are desirable. A multitude of imaging modalities can shine light on biological processes in bone tissue: for example, CT imaging can monitor structural details and bone remodeling at sub-millimeter resolution, PET/SPECT can be used to show metabolic processes \cite{1}, and MRI can help to diagnose fractures. Similarly, for preclinical studies in mice and rats, microCT, microSPECT, and high field MRI provide similar data. Since many of the bone imaging modalities rely on ionizing radiation, there is a large interest in finding new, non-invasive techniques suited for human application, especially for pediatrics and diseases which demand repeated imaging.

For ultrasound (US) imaging, bone used to be considered mainly as an obstacle due to its high acoustic impedance that causes a hyperechoic surface reflection with subsequent shadowing \cite{2}. In the last decades, this image has shifted, with increasing clinical applications for bone US. Compared to the bone imaging methods mentioned above, US has the advantage that it is non-invasive, fast, cost-effective and readily available. Especially in pediatric imaging, US has become a preferred method for many pathologies due to the above mentioned advantages and the easy access to US devices, which allows for parents to remain with their children \cite{3}. Studies on US imaging have shown its suitability to sensitively diagnose fractures in upper and lower limbs \cite{4-7}. Low frequency US is used to non-invasively measure bone material and structural properties in peripheral bones. This technique, referred to as Quantitative Ultrasound (QUS), was shown to have a comparable predictive power for fracture risk assessment in osteoporosis to the current gold-standard, Dual X-ray Absorptiometry (DXA). Correlations to bone mineral density (BMD) are significant but only moderate. Thus, there is still lack of evidence that QUS has the potential to replace DXA in the diagnosis of osteoporosis and the assessment of fracture risk \cite{8}.

\textbf{Abbreviations:} BMD, bone mineral density; DXA, dual-energy x-ray absorptiometry; FLI, fluorescence imaging; FMT, fluorescence molecular tomography; Hb, deoxygenated hemoglobin; HbO$_2$, oxygenated hemoglobin; ICG, indocyanine green; LDF, laser-doppler flowmetry; M, mean; NIR, near-infrared; PAI, photoacoustic imaging; PAT, photoacoustic tomography; QUS, quantitative ultrasound; RFU, relative fluorescence units; SD, standard deviation; SEM, standard error of the mean; US, ultrasound; % ID, percent initial dose; % PA signal, percent photoacoustic signal.

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In the preclinical setting, which is the focus of this contribution, bone imaging commonly includes optical modalities. A variety of bone-targeting fluorescence agents have been developed. Most notably, fluorophores are conjugated to hydroxypyaptite-binding bisphosphonates [9], but there are also smaller molecules combining fluorescence and targeting moieties (e.g. P80OS03) [10,11]. For example, a commercially available far-red fluorescent pamidronate derivative imaged by fluorescence molecular tomography (FMT) has been used to monitor physiological or pathological bone-turnover, both for osteolytic tumors and in osteoporosis [12,13]. The perfusion of bone flaps was demonstrated by intraoperative fluorescent imaging after perfusion with indocyanine green (ICG) in studies on cadavers, pigs and human patients [14–16] and similarly for teeth [17]. The first study to describe multimodal imaging of bone precursor cells within their physiological surrounding of the intact bone marrow space showed labeled and transduced osteoblastic cell lines that were implanted into the mouse femur and imaged by MRI and bioluminescence imaging [18].

Photoacoustic imaging (PAI) is a developing modality constantly expanding its application areas beyond the well-known imaging of lymph nodes or vasculature. A recent study has used PAI to assess the oxygenation status in a mouse femur in vivo, showing the possibility of imaging endogenous signals from the medullary space [19]. Superficial osteosarcoma lesions in rats were successfully imaged with PAI after contrast-enhancement with gold-nanorods [20]. Beyond these preclinical studies, PAI also has been shown to have some potential for translation to applications in humans. For example, single-wavelength PAI was suggested for guidance during spinal surgery based on an ex vivo study on vertebral specimen of healthy and cancellous bone [21]. Other studies showed that alterations in collagen and mineral content or mechanical properties could be measured by photoacoustics in human bone samples [22,23]. The translatability of PAI for clinical bone imaging will be further addressed in the discussion.

To date there are only few publications comparing photoacoustic tomography (PAT) and FMT in vivo. Recently, Chen et al. showed ex vivo that PAI compared to planar fluorescence imaging (FLI) has better sensitivity for signals deeper than 2 mm, but to the best of our knowledge, a validation in vivo is lacking [24]. In this study we investigated PAT as a novel method to detect intratibial signals as a non-invasive, non-ionizing, real-time imaging technique. Its performance was assessed in comparison to FMT, first ex vivo, in a phantom created to simulate the in vivo situation in intratibial injections. For in vivo comparison, ICG-containing liposomes were injected into mouse tibiae. ICG was chosen as a signal source, since it is a clinically approved near-infrared (NIR) fluorescent dye that also shows favorable photoacoustic properties. Liposomal carriers were created in order to avoid clotting of free ICG within the medullary cavity. In the fatty bone marrow the amphiphilic ICG molecule can bind lipids [25] and therefore a lower penetration would have been expected for the free agent. Additionally, lipid bound ICG shows a stronger signal in both FLI [26] and PAI [27]. The liposomes used in this study could potentially be filled with therapeutic or diagnostic agents and modified to target specific tissues or pathologies. The presented work therefore also lays the basis for preclinical theranostic studies targeting bone marrow or bone lesions. Such data, together with the innovative approaches discussed in the previous paragraph, should help to identify promising strategies for translation of PAI to human applications.

2. Material and methods

2.1. Liposomal nanoparticles

The manufacturing process and properties of the ICG liposomes were previously published [28], therefore the process will only be briefly described. Liposomes contained of DSPC/cholesterol/SM/DOTAP at 20:30:30:20 mol% (Avanti Polar Lipids, Alabaster, AL, USA) and were filled with ICG (Merck KGaA, Darmstadt, Germany) at a final concentration of 0.33 μg/μl inside the liposome. Lipids were solved in chloroform, pipetted to a glass tube, dried with nitrogen and lyophilized. ICG (1 mg/mL) and iron particles (5 nm Fe3O4, AC Diagnostics Inc., Fayetteville, AR, USA) in PBS were added to the tube and heated in a 60 °C water bath for 30 min. The solution was freeze-thawed and sonicated to create unilamellar liposomes. Purification was achieved by a Sephadex G-25 PD-10 column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

2.2. Phantoms for the determination of resolution and sensitivity

The phantoms were designed to resemble the physiological setting of the mouse tibia in vivo in order to assess and compare the capabilities and limitations of both modalities in a standardized model. The signal was fixed at 4 mm depth to account for the covering skin and muscle layers. A signal diameter of 0.75 mm was chosen to imitate the medullary cavity of BALB/c mice of the age used in the animal experiments. In order to evaluate the influence of the diameter of the cavity (and therefore injection volume) on resolution and sensitivity, a second set of phantoms with double the cavity diameter was created.

Tissue-mimicking phantoms were made out of 3 % agarose (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) with 4 % lipids (ClinOleic 20 %, Baxter International, Deerfield, IL, USA), in line with material previously described [29,30]. The 3D-printed positive molds were designed to leave defined cavities in the agarose blocks. For the determination of the resolution, 2 x five cavities with a diameter of 1.50 mm or 0.75 mm, were positioned at a distance of 5.0, 2.5, 1.0 and 0.5 mm. For the sensitivity, 2 x three cavities of 1.50 mm or 0.75 mm diameter were created at a fixed distance of 5 mm. After hardening, agarose blocks were carefully removed from the molds. The cavities were filled with ICG liposomes fixed in 3 % low-gelling temperature agarose (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The different diameters yielded a filling volume of about 10 μl (~3.3 μg ICG, ~425 μM) for the smaller holes (comparable to the tibia cavity) and 40 μl (~13.2 μg ICG) for the larger holes. For the sensitivity, the ICG liposomes were serially diluted in PBS by the factor 1:10 down to 0.1 % initial dose (%ID) and then fixed in low-gelling temperature agarose as described above. Images were obtained with a 4 mm layer of the tissue mimicking agarose described above covering the signal and repeated with three individual phantoms each.

2.3. Imaging of phantoms

Fluorescence image acquisition of all phantoms was performed with the FMT2500 (PerkinElmer Inc., Waltham, MA, USA) on the channel for 790 nm. The sensitivity phantoms were measured with the maximally offered sensitivity setting (termed “very high”) and a grid distance of 2 by 2 mm (“normal”). The resolution phantom was imaged at the default sensitivity level with a grid distance of 1 by 1 mm (termed “fine” in the software) with the transillumination facing onto the covered cavities, since perpendicular imaging showed diffuse, less well-defined signals. The resulting imaging depth was 4 mm, which was the same as for longitudinal imaging. FMT images were manually optimized after acquisition in order to find a threshold that would lead to the best possible signal discrimination. The resulting thresholds were kept constant between phantoms of the same diameter, but differed between the diameters. VOIs were fitted onto the cavities and into the background.

PAI was performed on the Vevo 2100 ultrasound system combined with a Vevo LAZR (both FUJIFILM VisualSonics Inc., Toronto, CA). The resolution was assessed in PAI by measuring the respective phantoms at multiple wavelengths (680 nm, 700–950 nm in 25 nm steps, 970 nm), at a distance of 10.15 mm with a step size of 32 μm. The sensitivity of PAI was determined in two-dimensional transverse spectral scans (680–970 nm, step size 5 mm) by the signal intensity at 820 nm (peak of liposomal ICG) in ROIs around the cavity versus background.
2.4. Animal experiments

All animal experiments had been approved by the local animal ethics committee (MELUR, V242–62132/2016 (122–10/16)). Animals were housed in temperature-controlled rooms with 12 h light/dark cycle and access ad libitum to water and standard rodent food before the study. Ten 2–6-week old BALB/c mice were anesthetized with 75/0.5 mg/kg body weight ketamine/medetomidine. The fur of both legs was carefully removed by depilation cream to avoid rashes or cuts. The maximum duration of all imaging procedures was one hour. The animals were then sacrificed by cervical dislocation while still under anesthesia.

Mice legs were imaged with the FMT2500 on the 790 nm channel with the standard sensitivity setting and a 2 × 2 mm grid distance, leading to an image acquisition time of about 5 min. Mice were positioned in the holder in the lateral position with the respective leg facing up. The scan area was bordered distally below the ankle joint and proximally above the knee region (Fig. 3A).

Transverse three-dimensional PAT of a comparable region of the proximal tibia was performed on the Vevo 2100/Vevo LAZR at multiple wavelengths (680 nm, 700–950 nm in 25 nm steps, 970 nm) and a step size of 216 μm. The animal was placed on a heated table in the lateral position with the respective leg facing up on a small roll of gauze in order to put the bone in a horizontal imaging plane parallel to the transducer. This measure was taken to facilitate a reproducible set-up and achieve a constant energy deposition. Centrifuged and pre-warmed ultrasound gel was generously applied to the leg. The scan area for the PAT was determined in B-mode ultrasound with the starting position just below the knee joint (Fig. 3). Additionally, a two-dimensional spectral scan (wavelengths 680–970 nm in 5 nm steps) in the transverse plane of the proximal tibial corpus was performed.

After background imaging in both modalities, about 10 μL of ICG liposomes (3.3 μg ICG) were injected into the left or right tibia by the parapatellar approach. Then all imaging modalities were repeated as described above. The order in which PAT and FMT were performed (background and after injection) was altered between mice to avoid a bias based on timing. Mice were killed by cervical dislocation. Both tibiae were excised and cleaned of remaining muscle tissue. Imaging was again repeated with the excised mouse bones placed on a tissue mimicking phantom for FMT and in a water bath for PAT.

2.5. Image and statistical analyses

FMT images were analyzed with the TrueQuant Software Version 3.1 (PerkinElmer Inc., Waltham, MA, USA) by fitting a VOI including the maximal signal within the tibia region. PAT images were analyzed with the Vevo LAB analysis software version 1.7.2 (FUJIFILM VisualSonics Inc., Toronto, CA). The three-dimensional multi-wavelength scans were spectrally unmixed for ICG liposomes versus blood and a VOI was fitted closely around the bone in the ultrasound images. The spectral scans were evaluated by placing an ROI around the tibia outline and an equally sized copy into the muscle tissue.

Statistical analyses were carried out with Microsoft Excel, GraphPad PRISM, ImageJ 1.8.0 and JMP by SAS Institute. The mean fluorescence (relative fluorescence units, RFU) or percent photoacoustic (%PA) liposomal ICG signal in the tibia before and after the injection was compared by Mann-Whitney for non-parametric or student’s t-test for parametric data. Correlation between modalities and between in vivo and ex vivo imaging within each modality was tested with Spearman’s rank-order or Pearson correlation. Sensitivity of FMT and PAT was compared using a paired Wilcoxon signed rank test. For a more precise comparison in the standardized sensitivity phantom, the intensity at 820 nm of the circled signal in the 2D transverse spectral scan was used in PAL.

3. Results

3.1. Phantom studies

The results of the phantom study are depicted in Fig. 1 (resolution) and Fig. 2 (sensitivity). All following results are referring to a signal depth of 4 mm and the above described ICG liposomes. The phantom matrix itself did not cause any signal in either fluorescence or photoacoustic imaging. For the FMT, the 0.75 mm diameter signals fused at a distance of 1 mm (Fig. 1C), signals with 1.5 mm diameter at 0.5 mm distance (Fig. 1D). The sensitivity in FMT was limited to 3.33 ng/μl ICG (1 % ID, 33 ng ICG, p = 0.0207 versus background) for the cavities resembling our in vivo experiment (Fig. 2A), and to 33 ng/μl ICG (10 % ID, 1.32 μg ICG, p < 0.003 versus background) for the larger cavities (Fig. 2B). The signal intensity was generally higher in the larger cavities, but signals were more diffuse with a higher background. The lower threshold limit therefore needed to be higher for the larger cavities, than for the smaller diameter. Results were similar for an isolated test of the lowest concentration without background thresholding (data not shown). In the ultrasound images, that were acquired simultaneously to the PAT, the cavities showed a different echo attenuation compared to the surrounding phantom matrix. These apparent inhomogeneities generally caused no photoacoustic signal. In both FMT and PAT, the size of the depicted signals decreased with the ICG concentration. Only 10 % ID lead to the depiction of the true point source diameter in all phantoms and both modalities. The PAT showed all individual signal sources in the resolution phantom independent of the signal diameter, which implicates a nominal resolution limit below the tested minimum of 500 μm (Fig. 1E and F). Unfortunately, the 3D-printer could not produce smaller distances, so a resolution limit could not be determined for PAT. The PAT system detected all signals of both diameters (0.75 and 1.50 mm) down to the lowest concentration of 0.33 ng/μl ICG (0.1 % ID, 3.3 or 13.2 ng ICG respectively) (Fig. 2C and D).

3.2. Animal experiments

The background images in the FMT showed no to very low fluorescent signals (M: 1.39 RFU, SD: 2.63, SEM: 0.83) (Fig. 4A, C). After intratibial injection of ICG liposomes, the signal significantly increased both in the in vivo measurement (M: 9443 RFU, SD: 4023, SEM: 1272, p < 0.0001; n = 10) (Fig. 4B, C) and ex vivo (M: 9456 RFU, SD: 10322, SEM: 3264, p < 0.0001) (Fig. 4C). Due to limited spatial resolution of FMT, the signal covered both the tibia and the muscles in the vicinity. For PAT, tibia also showed a low endogenous background signal, which could be further reduced by spectral unmixing (M: 0.62 %, SD: 0.91, SEM: 0.29) (Fig. 4D, G). After intratibial injection, there was a significant increase in percentage of PA signal in the VOI fitted to the tibia (M: 42.48 %, SD: 23.12, SEM: 7.31, p = 0.0003, n = 10) (Fig. 4E and G). The photoacoustic signal was located in the tibial head and inside the medullary cavity in the proximal tibial body. Also, small signals appeared on the skin line (Fig. 4E). The background spectrum inside the tibia had a main peak at 750 nm, followed by a steep increasing tendency between 790 nm and about 930 nm (Fig. 4F). The spectral scan after the injection showed that the photoacoustic signal exhibited the expected peak at the absorption maximum of liposomal ICG (Fig. 4F). A comparison of the changes after ICG injection of FMT (Fig. 4C) vs PAT (Fig. 4G) based on paired comparisons did not reveal consistent advantages for either technique: Wilcoxon signed rank test results varied depending on whether FMT and PAT results were standardized by standard deviation or interquartile ranges.

In vivo measured signals after the intratibial injection of ICG liposomes correlated strongly and significantly with the ex vivo signals in the excised bones in both FMT (R² = 0.76, p = 0.0010, n = 10) (Fig. 5C) and PAT (R² = 0.70, p = 0.0024, n = 10) (Fig. 5E). Comparison between modalities also showed a moderate but significant correlation between
the in vivo signals after injection ($R^2 = 0.69$, $p = 0.0030$, $n = 10$) (Fig. 5B) and between the ex vivo signals in the excised bones ($R^2 = 0.58$, $p = 0.0110$, $n = 10$) (Fig. 5F).

4. Discussion

Our results demonstrate that ICG labeled liposome accumulation in the tibia of mice can be visualized with FMT and PAT, showing the suitability of both techniques for preclinical in vivo studies. For most of our data, PAT showed clearly superior performance compared to FMT, specifically for spatial resolution, also for sensitivity. These results apply to an imaging depth of 4 mm and when the here described ICG liposomes are used as a contrast agent.

The results of the phantom study shown in Figs. 1 and 2 support the hypothesis that PAT can depict intratibial signals from our specific ICG liposomes with a resolution and sensitivity superior to FMT. Depending on the experimental setup, FMT could only resolve structures above $0.5–1$ mm. The resolution limit determined thus was more than ten-fold lower than the minimal image pixel resolution of 156 mm in the technical specification sheet of the manufacturer [31]. A smaller grid distance may improve the resolution, but would increase the scan time exponentially, since half the grid distance leads to a squared number of imaging points. Also, the manufacturer recommends 35–75 sources for optimal results and a maximum of 120 point sources, which would have been exceeded with an even finer grid. Resolution results obviously also depend on scatter, the geometry of the phantom and the chosen contrast agent. PAT showed a nominal spatial resolution better than 0.5 mm at the implemented signal depth of 4 mm. Our results are in agreement with a previous study by Chen et al. using a tissue mimicking phantom, that came to the conclusion that at a depth $> 2$ mm PAI shows a better sensitivity and resolution than FLI [24]. Chen et al. imaged different fluorescent dyes, including ICG, at a signal depth of 0–12 mm and with four different concentrations (100, 50, 10 and 2 $\mu$g/mL compared to 330, 33, 3.3 and 0.33 $\mu$g/mL in our study) through tissue mimicking medium and mouse tissue by PAI and planar FLI. For a simulation of in vivo imaging, they placed a tubing containing ICG solution under the thigh of a mouse. Another comparative phantom study between PAT and FMT used ICG in agarose phantoms up to a depth of 7 mm and concluded, that while PAT has a higher spatial resolution, the sensitivity is superior for FMT [32]. This study by Wang et al. used three different ICG concentrations (100, 10 and 2 $\mu$M compared to 425, 42.5, 4.25 and 0.425 $\mu$M in our study) for sensitivity and distances of 2, 4 and 6 mm with a constant concentration of 100 $\mu$M ICG for resolution studies. Differences to our study are mainly the instruments used, since both Chen et al. and Wang et al. performed experiments on custom made instruments, while our study compared commercially available devices with the original manufacturer’s software and can therefore easily be reproduced. The other important distinction of our study is that both studies mentioned above did not acquire in vivo data, which was the main motivation for this investigation. A limitation to both our phantom study and previous comparable phantom studies is the lack of a bone-mimicking phantom. The specific challenges of penetrating soft tissues and bone material with either modality were not simulated, which makes a prediction of imaging for example through thicker bone difficult. Moreover, results regarding resolution and sensitivity could vary for different contrast agents. A higher quantum yield compared to
IGC could improve especially the sensitivity of fluorescence imaging. Future studies should therefore investigate different contrast agents. We limited our study to tomographic modalities, in order to facilitate a more direct comparison. The FMT 2500 uses transillumination for the three-dimensional reconstruction of signal origin. Projection fluorescence imaging systems, like the IVIS by Perkin Elmer, are supposed to achieve a comparably higher sensitivity. Both projection and planar imaging, as described by Chen et al. [24], might therefore achieve or even overcome the sensitivity that we determined for our phantoms in the FMT2500, which would explain our partially discrepant results. Another shortcoming is the failing reconstruction of the full signal diameter in the sensitivity phantoms containing lower liposomal ICG.

Fig. 2. Sensitivity of FMT and PAT assessed on agarose phantoms. Average signal intensity (mean ± SEM) of signals and background in sensitivity phantoms containing 0.1 %, 1 %, 10 % of initial dose from undiluted ICG liposome solution (33 ng/μl ICG), n = 3 with signal diameter 1.50 mm (B, D) and 0.75 mm (A, C) respectively. A: average mean fluorescent signal of a signal with 0.75 mm diameter with example of FMT image with background photo (grey) and fluorescence image (color scale); B: average mean fluorescent signal of a signal with 1.50 mm diameter; C: average PA signal at 820 nm of a signal with 0.75 mm diameter and example of US image (grey) with PA signal after spectral unmixing (red); D: average PA signal at 820 nm of a signal with 1.50 mm diameter.

Fig. 3. Orientation of measurements in FMT, US and PAT. A: Example background photo of mouse in FMT positioned on the side with 3D area for FMT (grey cube), US (white cube) and PAT (red cube). While the FMT merges sagittal images, the position of the US/PA transducer is perpendicular merging transverse slices for 3D tomography. B: Acquisition set-up of 3D US/PAT images.
concentrations. Signals were expected to show a decreasing signal intensity with reduced concentrations, but should still remain in the geometrical shape of the source. Instead, the depicted signal area decreased along with the ICG liposome concentration in both modalities. Both reconstruction algorithms appear to be challenged when concentrations approach the sensitivity limit. In the \textit{in vivo} setting, the dependence of signal area on signal concentration would interfere with a correct spatial resolution. A further investigation should therefore investigate, whether this effect occurs \textit{in vivo}. Potentially, other factors, like binding to local proteins (e.g. fat, albumin), active or passive accumulation increase the signal \textit{in vivo} sufficiently to avoid these effects.

Our \textit{in vivo} data shown in Fig. 4 supports that both FMT and PAT are modalities suitable for the visualization of signals from the medullary cavity. Despite the better performance of PAT in these specific phantom studies, no consistent difference in technique sensitivity could be observed \textit{in vivo}. One reason may be the naturally low endogenous background of near infrared fluorescent contrast agents. Raw PAT images on the other hand show some background signals. These signals are likely caused by the highly vascularized bone marrow due to the overlap in the photoacoustic spectra of liposomal ICG and blood. This makes a complete spectral unmixing challenging. Concordantly, the background spectral scan of the tibia exhibited a peak at 750 nm, typical for deoxygenated hemoglobin (Hb) and a second signal increase towards 850 nm, as expected from oxygenated hemoglobin (HbO$_2$, Fig. 4F, dashed black line). The same can be seen in the PA spectrum of the

Fig. 4. FMT and PAT imaging before and after intratibial injection of ICG liposomes. A and B: Representative images for FMT before (A) and after (B) ICG liposomes were injected; D and E: Representative spectrally unmixed images for PAT before (D) and after (E) injection of ICG liposomes. F: Analysis of PA spectroscopy before (dashed lines) and after (continuous lines) ICG liposomes were injected in the muscle (blue) and tibia (black); the white dashed line in D and E indicate the imaging plane for respective transverse 2D spectroscopic scans. Quantitative statistical evaluation demonstrates that both FMT (C) and PAT (G) yield ICG signals from the medullary cavity that are significantly larger than the background; this could be confirmed on isolated bones \textit{ex vivo} ($n = 10$).

Fig. 5. Correlation between FMT and PAT signals measured \textit{in vivo} and \textit{ex vivo}. Representative sagittal \textit{in vivo} (A and D, left) and \textit{ex vivo} images (A and D, right) for (A) FMT and (D) PAT. Quantitative data shows significant intra- (C, E) and inter-technique (B, F) correlations \textit{in vivo} and \textit{ex vivo}.
muscle before and after ICG liposome injection (Fig. 4F, blue lines continuous and dashed). The intratibial signal after ICG liposome injection exhibits a broad peak roughly between 790 and 850 nm that overlaps with both hemoglobin peaks, but reaches its maximum at about 820 nm (Fig. 4F, continuous black line). The absorption of strong biological absorbers, like whole blood, may largely exceed the absorption of smaller amounts of ICG. This does not necessarily interfere with either the photoacoustic or fluorescent ICG signal, since the amphiphilic molecule readily binds to albumin. Similar to lipid binding, the albumin binding leads to an increase in signal intensity [33]. However, the peak-specific intensity increase in our animal study was sufficient to allow for a successful discrimination between ICG liposomes and the surrounding vascularized tissue based on spectral unmixing. Skin signals (Fig. 4E) were only present after the ICG liposome injection, indicating that they show the injected contrast agent. The skin signal could represent contrast agent that has already entered circulation and perfuses the dilated vessels in the depilation area. The signal might additionally be enhanced due to an increased local blood flow, since a slight irritation cannot be avoided during chemical depilation. The correct depiction of ICG liposomes in PAT was confirmed by the comparison to FMT in vitro, in vivo and ex vivo. Despite the yet inconclusive data on sensitivity the better spatial resolution represents a clear advantage of PAT measurements in vivo, in comparison with FMT.

Ex vivo imaging of excised and cleaned bone samples and the correlation of ex vivo signals with corresponding in vivo measurements confirmed the signal origin and quantification. The programs used in this study for FMT analyses and PAT analyses work differently and this needs to be considered when interpreting the findings. In the FMT scans, VOIs cannot be fitted tightly to the bone, mainly because it is not visible, but also because the software supplies only three pre-determined geometrical VOI shapes. As an outcome, we selected the mean fluorescence signal of a VOI of consistent size for analyses in vivo and ex vivo. While for scans in vivo additional signals from the surrounding tissue were included, this was not the case ex vivo. These additional signals that occur after the injection are most-likely from scattering of localized signal into the surrounding area, as suggested by the increasing signal area in in vivo versus ex vivo scans (Fig. 5A). Another explanation for background signals in FMT after the injection could be the starting circulation of the contrast agent. The observation is consistent with skin signals appearing in PAT after injection, that were already described above. This effect would explain the observed reduction of the slope to 0.74 ± 0.15 (Fig. 5C) but the coefficient of determination of $R^2 = 0.76$ was still fairly strong. In PAT, however, the bone can be determined in the US scan. This permits the selection of a tightly fitting VOI, that excludes possibly leaked contrast agent. Indeed, the slope of results in vivo versus ex vivo does not differ significantly from 1.0. At $R^2 = 0.70$, the coefficient of determination is similar to that observed for FMT and, interestingly, also to that observed for the inter-modality association of $R^2 = 0.69$.

The PAT software also has some limitations. For the version of Vevo Lab used to obtain the PAT scans in this study only %PA signal could be determined, using a voxel-based thresholding algorithm calculating the percentage of voxels with a signal exceeding the threshold among all voxels in the image. Thus, signal strength within a voxel is only partially evaluated. Later versions of the instrument offer more detailed parameters (e.g. average signal), that would be more similar to the method used for FMT VOI analysis.

The PAT signal shows that the correlation between the modalities is also affected by the different physical effects of FLI and PAI. Both modalities initially depend on the molar extinction coefficient, the length of the optical path and the concentration of the contrast agent, according to the Beer-Lambert law for absorbance. Fluorescence signal intensity is additionally dependent on the quantum yield of the contrast agent [34], whereas the photoacoustic response depends on the thermoelastic expansion coefficient [35]. Technical parameters such as light source intensity and detector efficiency are additional instrument specific factors that can influence the measured signal intensity. Considering all of these differences between factors affecting signals of FMT and PAT the observed level of 69 % of the results of one modality being explained by the other one is actually quite strong. A comparison of the magnitude of the signal levels of the two modalities is difficult, given the different unit of measurements. Still, earlier studies have reported that the signal increase compared to control is higher in FLI than in PAT (FLI/PAI: 8/-2-fold, Atto 740-labelled peptide [36], 6/-3-fold, ICG-labelled peptide [37], both targeting tumor surface markers), which is consistent with our results if we would disregard normalization. It is more meaningful to evaluate sensitivity of pre versus post contrast signals and to normalize the results by interquartile ranges. For this study, the sample size was too limited to yield definite answers to this question.

Both FMT and PAT showed variations across mice in the extent of the signal localization and intensity. Factors contributing include the penetration depth of the contrast agent into the tibial shaft and a relatively high variability in intratibial injections. Although potential technical pitfalls in intratibial injections have rarely been described, it is known that especially the curvature and the limited space in the bone marrow cavity may cause leakage from intratibial injections [38], making the procedure less standardized compared to an intravenous injection. PAT can present a method for quality control in studies with intratibial injections, as Fig. 6 demonstrates. The left panel shows an example of a successful intratibial injection, while in the right panel a large part of the injected volume has either leaked out of the medullary cavity or was wrongfully injected on top of the bone (the depicted mouse was excluded from the study). The difference is obvious in PAT, while the images are comparable in FMT.

Our study has strengths and limitations. A main strength of this study is that individual animals were subsequently subjected to both imaging modalities (in random order). This allows for a direct comparison and is especially important considering the inter-individually varying injection volumes and the possibility of liposomes circulating or leaking out of the bone marrow space over time. A major limitation of this study is the injection method. As described above, intratibial injections have challenges, leading to the question whether the results of this study have any implications for intravenously or intracardially injected contrast agents, drugs or cells. In this model, we injected ~10 μL of ICG liposomes, which is about 5 % of the maximally possible intravenous injection volume in mice (up to 10 % of blood pool volume, ~200 μL). According to Sou et al., only 1–2 % of non-targeted liposomes are retained in the bone marrow, yet bone marrow targeting can increase the local uptake to 12.5 ± 1.5 % injected dose [39]. Our own liposomes can also be modified to target the bone reconstruction sites via alendronate [40]. The sensitivity phantom study implies that lower concentrations of ICG liposomes could also be visualized by PAT. Additionally, the liposomes concentration in the injection solution could be increased about five-fold. It is difficult to make a final estimate about the signal levels to be expected for in vivo studies employing intracardial or intravenous application of liposomes labeled with ICG. However, we consider this to be feasible and prospective validation studies are warranted. Such studies would be of substantial interest in the context of disease models such as osteoporosis or bone metastases.

Our study shows that PAT facilitates high-resolution, non-invasive, real-time imaging of the bone marrow space in mice. The established method could therefore be used to assess the pharmacokinetics and biodistribution following an intravenous injection, especially of a lipophilic agent, in a future study. Due to their size-dependent character and cationic properties, an interaction of liposomes with bone marrow cells, like adipocytes or osteal macrophages, can be expected and would largely influence retention, release and circulation time of the enclosed cargo.

Further studies should also address the question whether the here presented technique for non-invasive bone imaging by PAI could be translated into the clinic in the future. The assessment of marker signals for the tibial cavity is relevant in a number of disorders such as bone
metastases, multiple myeloma, pathologies of the lipid metabolism and immune responses. However, given the strong absorption of photons and ultrasound signals in bone, it remains to be investigated whether fluorescent or photoacoustic signals from the marrow space can be detected and quantified in human patients. One particular application that would be of interest is the monitoring of drug delivery to the bone marrow, e.g. by drug containing labeled liposomes [41]. A major limiting factor for both PAI and FLI in the clinical setting is the very limited number of both approved contrast agents and specifically targeted contrast agents. FLI and PAI depend on the penetration of excitation light into the bone, which requires the bone to be rather superficial and thin. Generally, light propagation into tissue is limited to several centimeters [42]. Interestingly, Henderson and Morries reported an increased penetration depth of pulsed NIR light into tissues that contained bone compared to boneless subcutaneous tissue in vivo human hands [43]. Binzoni et al. demonstrated that they can non-invasively measure the pulsatile blood flow in the clavicle, patella, tibial malleolus and diaphysis by laser-Doppler flowmetry (LDF) at 800 nm in healthy human patients, showing that light can penetrate a variety of human bones [44]. In contrast to LDF both FLI and PAI generate their response signal inside the tissue as photon emission or ultrasound, respectively. The second challenge to measurable signals is therefore creating a signal that can reach the respective detector. As mentioned before, PAI has the capability to penetrate ex vivo human bone samples, but in vivo data is still lacking. Since a penetration depth of about 2–3 cm can be achieved with clinically-approved PAI systems [45], many bones, like the skull, radius or tibia could potentially be imaged in humans.

5. Conclusions

In this study, we showed that non-invasive PAT monitoring of ICG labeled liposomes in the murine bone marrow cavity is feasible in vivo. Compared to FMT, PAT demonstrates superior spatial resolution both on phantoms and in vivo at an imaging depth of 4 mm with the described ICG liposomes. Sensitivity is also higher for PAT in the studies on these specific phantoms. Further studies using the latest software options are required to assess whether this also translates into studies carried out in vivo. PAT and FMT could both be used for multimodal studies on bone metastases, targeted drug delivery, bone marrow-derived diseases and bone physiology and our results demonstrate that further prospective investigations in vivo are warranted.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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