A temporal and spatial analysis approach for automated segmentation of microbubble signals in contrast enhanced ultrasound images – application to quantification of active vascular density in human lower limbs

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Abstract

Contrast enhanced ultrasound (CEUS) using microbubble contrast agents has shown great promise in visualising and quantifying active vascular density. Most existing approaches for vascular density quantification using CEUS are calculated based on image-intensity, and are susceptible to confounding factors and imaging artefact. Poor reproducibility is a key challenge to clinical translation. In this study a new automated temporal and spatial signal analysis approach is developed for reproducible microbubble segmentation and quantification of contrast enhancement in human lower limbs. The approach is evaluated *in vitro* on phantoms and *in vivo* in lower limbs of healthy volunteers before and after physical exercise. In this approach vascular density is quantified based on the relative areas microbubbles occupy instead of their image intensity. Temporal features of the CEUS image sequences are used to identify pixels that contain microbubble signals. A microbubble track density (MTD) measure, the ratio of the segmented microbubble area over the whole tissue area, is calculated as a surrogate for active capillary density. In vitro results show a good correlation (r^2 = 0.89) between the calculated MTD measure and the known bubble concentration. For in vivo results, a significant increase (129% in average) in the MTD measure is found in lower limbs of healthy volunteers after exercise, with excellent repeatability over a series of days (ICC = 0.96). This compares to the existing state-of-art approach of destruction and replenishment analysis on the same subjects (ICC <= 0.78). The proposed new approach demonstrates great potential as an accurate and highly reproducible clinical tool for quantification of active vascular density.

Key words: contrast enhanced ultrasound, lower limb, vascular density quantification, image segmentation, temporal analysis, reproducibility, peripheral arterial disease

Introduction

Ultrasound is a safe, affordable and accessible front-line clinical imaging modality, characterised by real-time image display. Recent advances in contrast-enhanced ultrasound (CEUS) imaging, provide the possibility of specifically imaging blood vessels with high sensitivity and resolution. Microbubbles move through the body while being confined to blood vessels, distinguishing them as an excellent intravascular contrast medium. They vibrate under ultrasound and in a non-linear fashion, generating specific harmonic signatures that allow them to be distinguished from background tissue signals with a high sensitivity.

CEUS is ideally suited for measurements of flow and vascular density, as bubbles move within the blood vessels at comparative speeds to blood cells. A destruction-replenishment approach has been used in many in vitro and in vivo trials with success. High amplitude ultrasound is used to destroy microbubbles within the imaging plane, then the replenishment of the region is observed over time. To quantify vascular density, Time Intensity Curve (TIC) analysis is conducted to extract a number of physiological parameters such as peak intensity and flow rate etc. This method estimates parameters related to vascular characteristics of the tissue and has been applied to the study of liver (Claudon, et al. 2013) and heart (Senior, et al. 2013, Wei, et al. 1998). Recent studies have shown particularly great promise in evaluating neovascularisation in atherosclerotic plaques (Hellings, et al. 2010, Huang, et al. 2008, Xiong, et al. 2009), the myocardial microcirculation (Senior, et al. 2013, Wei, et al. 1998) and the musculoskeletal microcirculation of the lower limb (Amarteifio, et al. 2013, Amarteifio, et al. 2011, Duerschmied, et al. 2009, Krix, et al. 2011, Krix, et al. 2009, Lindner, et al. 2008, Mitchell, et al. 2013, Song, et al. 2014).

However the quantification of vascular density using CEUS is affected by many confounding factors (Tang, et al. 2011). In particular most existing analysis is image-intensity based, and such an approach is vulnerable to problems such as signal attenuation, and nonlinear imaging artefacts (Cheung, et al. 2015, Yildiz, et al. 2015). An alternative approach to individual bubble tracking and quantification within the image have been reported, particularly in peripheral imaging applications where relatively high frequencies are commonly used (4-15MHz). While imaging with such frequencies reduces sensitivity in bubble detection (Tang and Eckersley 2007) and only the brightest bubbles show up in the CEUS images, the improved spatial resolution associated with such high frequency could facilitate the tracking of individual bubbles. Hoogi et al. (Hoogi, et al. 2012) proposed a method for segmenting the contrast spots within atherosclerotic plaques in individual images by tracking individual microbubbles. The main advantage of this approach is that the temporal behaviour of bubble flow can be demonstrated. This makes it robust to noise and allows differentiation between blood vessels and artefacts.

Recently several groups have developed various methods for single bubble detection and tracking by taking advantage of some temporal information. Viessmanns et al. and Christensen-Jeffries et al. used rolling background subtraction to remove unwanted background signals from static structures such as the echo from the tube wall (Christensen-Jeffries, et al. 2015, Viessmann, et al. 2013). Ackermann et al. adopted a temporal median filtering and foreground/background subtraction to detect and track of multiple microbubbles in ultrasound B-Mode Image (Ackermann and Schmitz 2016). Errico et al. developed ultrafast ultrasound localization technique for deep super-resolution vascular imaging by exploiting the coherence of backscattered signals, the spatiotemporal filtering approach discriminates slowly moving bubbles of sub-wavelength size (low spatial coherence) from slow motion tissue signals whose

temporal variations affect many neighbouring pixels the same way (high spatial coherence) (Errico, et al. 2015). Gessner et al. developed acoustic angiography to visualise microvascular architecture without significant contribution from background tissues by using super-harmonics and a customised dual-frequency probe (Gessner, et al. 2013). Mischi et al. used spatiotemporal analysis of ultrasound contrast agent dispersion kinetics to image angiogenesis (Mischi, et al. 2012). In this study we propose a different method and apply it to a clinical application of quantifying active vascular density in human lower limbs. Comparing to the existing techniques, the proposed method examines frequency features in the temporal domain which is image intensity independent and hence may be more robust to the various confounding factors such as attenuation."

In CEUS image sequences, we hypothesise that the temporal profile of each pixel can be used to detect microbubbles passing the pixel. The relative area of these "bubble pixels" can provide an area-based vascular density measure that may be more robust than existing image-intensity based approaches. Furthermore, the pixel-based temporal analysis can be reduced to an automated algorithmic process, giving advantages in terms of user interface, output speed and interpretation over existing approaches.

The objective of this study was to develop a robust and automated quantification tool for microbubble activity in CEUS image sequences using a pixel level temporal and spatial analysis based algorithm. This technique will be demonstrated with a flow phantom and then, as an initial clinical demonstration, applied to the quantification of in vivo musculoskeletal microcirculation in lower limb vascular density of healthy human subjects.

Materials and Methods

Microbubble detection algorithm

The proposed algorithm works at a pixel level to detect microbubble signals. The image contains primarily three components: tissue artefact, noise, and microbubble signals. Initially, average image intensity and coefficient of variation are used to remove tissue signals, and then microbubbles are distinguished from noise by examining the frequency composition of the pixel's temporal signal. The temporal signal of a pixel within a vessel with bubble(s) passing through has very different frequency composition from that with noise only (See Figure 1). The microbubble detection algorithm consists of the following specific steps.

1) Detecting tissue only regions.

Given the signal, *I(t)*, the coefficient of variation (COV) is shown as follow,

$$COV = \frac{\sqrt{\langle (I(t) - \langle I(t) \rangle)^2 \rangle}}{\langle I(t) \rangle} \quad (1)$$

where $\langle I(t) \rangle$ is the temporal average intensity. If we assume tissue signals to be higher in amplitude than noise background and not changing significantly over time, the combination of COV and average intensity can be used to identify tissue signal. If a signal's COV is smaller than a threshold T_{COV} and its average intensity is larger than a threshold T_{AI}, this signal is classified as tissue signal. The threshold values of T_{COV} and T_{AI} are estimated empirically by examining the histograms of the datasets. Based on the parameters, COV and average intensity described in the method section, their thresholds were set in order to detect and separate tissue signals. Before bubbles flowed through the target ROI, the intensities of tissue and noise could be estimated by analysing in these pre-contrast frames manually selected regions of tissue and

noise. They were used as a template for threshold selection. The corresponding threshold values for COV and average intensity were determined by finding the intersection of tissue and noise distributions in the parameter histogram. The combination of COV and average intensity can be used to identify tissue signal. The remaining unclassified signals contain microbubbles and noise.

Figure 2A and 2B show screen captures from a human subject's gastrocnemius after an intravenous injection of Sonovue. It can be seen that microbubble signals, tissue signals (arrows in Figure 2A) and noise are visible in the image.

2) Separating microbubble regions from regions of noise through examining temporal features

It is assumed that the temporal noise of the ultrasound data is white noise (Bar-Zion, et al. 2015, Barrois, et al. 2013) and hence broadband. For a pixel where a microbubble(s) passed through, the temporal signals are expected to have more low frequency components depending on the velocity of the microbubbles. Therefore a simple way to identify microbubble signal from noise is to look at the frequency features of the signals.

Example time intensity curves (Figures 1A and 1C) and their spectra (Figures 1B and 1D) for microbubbles and noise from single pixels of in vivo human data are shown. It can be seen that the microbubble signal consists of more low frequency components, while the noise is spread over the whole spectrum, thus allowing their separation. In this study, we fix the time window for Fourier analysis to be 30 seconds. This is empirically chosen in order to generate reasonable amount of segmented bubble signal within the image plane.

Before describing the following steps of the method, the physiological relevance of the frequency of the microbubble signal should be explained. The rate of change of the intensity at a point is related to flow velocity. Therefore, given a single microbubble with velocity $v_b = \frac{d}{t}$, where the *d* is the distance travelled by it in time *t* either within or across the ultrasound imaging plane. For a certain concentration of microbubbles, if we assume that the microbubbles are well mixed and the average separation distance of two neighbouring microbubbles is *D*, while the duration between one bubble passing a certain pixel and its neighbour bubble passing the same pixel is *T*, the velocity of a single microbubble can be described by equation (2):

$$v_b = \frac{D}{T} = fD \tag{2}$$

where *f* is the inverse of *T*, i.e. a frequency. Assuming a constant concentration, the frequency is linearly related to the velocity of microbubbles.

While the frequency is determined by microbubble velocity, it is also affected by microbubble concentration and other factors. To improve the robustness of the method, instead of examining the fine features on the spectrum, a simple measure of relative weighting of the signals high and low frequency components is used in this study. Given the microbubble signal, $I_b(t)$, noise signal, $I_n(t)$, and their power spectra $\widehat{I_b}(f)$, $\widehat{I_n}(f)$, a cut-off frequency, f', is defined (see equation (3)) to separate the spectrum into low and high frequency regions. The area under curve is then calculated (exclude the DC component) for these two regions correspondingly. A high-to-low frequency ratio (HLFR) is calculated:

$$HLFR = \frac{\sum_{f>f'}\left|\hat{I}(f)\right|}{\sum_{f\leq f'}\left|\hat{I}(f)\right|} \tag{3}$$

The ratio is used to classify a given signal as either microbubble or noise. For a pixel containing e.g a microvessel, as microbubbles occasionally pass this otherwise dark pixel, its temporal

signal is expected to have a higher proportion of lower frequency components than white noise. Consequently the HLFR of the pixel is expected to be smaller than that of noise.

A histogram of normalised HLFR for each CEUS image sequence is then constructed where two peaks are expected (See Figure 3), one corresponding to microbubbles and the other to noise background. A HLFR threshold, T_{HLFR}, is then determined to separate the microbubble and noise distribution. To automatically determine the threshold, the histogram is fitted using a double-Gaussian model. The threshold is set at the interception of these two Gaussian distributions (Otsu 1979).

The cut-off frequency f' in equation (3) to separate the high and low frequency components in the signal spectrum is important and needs to be optimised. We formulate an optimisation solution to estimate the optimal cut-off frequency, $\widehat{f'}$, by maximising the distance between the bubble peak and noise peak, $L(f')$, in the HLFR histogram,

$$\widehat{f'} = arg\,\max_{f'}(L(f')) \tag{4}$$

Once the optimal $\widehat{f'}$ is determined, the threshold T_{HLFR} can be computed accordingly to segment out bubble areas. The normalised histogram is calculated from HLFR. The value of HLFR is normalised by the maximum HLFR within the ROI. The peak positions and the distance *L* are taken from the fit of two Gaussian distributions for a given cut-off frequency f'. The optimal cut-off frequency $\widehat{f'}$ is determined by an iterative procedure (optimisation). Given that there are only two types of signals, microbubbles and noise, the following constraints are set in order to obtain a valid solution. (1) Two peaks should exist and be positive; any negative peak is considered as an unphysical solution and therefore, is rejected. (2) There must be an

intersection between two peaks. We then choose the optimal cut-off frequency $\widehat{f'}$ such that the distance L is maximised.

Finally a spatial filtering is conducted to the segmented image to remove isolated pixels of noise to further improve the robustness of the algorithm. A 3x3 pixel median filter is applied. The size of the filter is determined when taking into account the spatial extent of a microbubble in an image.

Microbubble track density (MTD) measure

The number of pixels identified as having bubble signals is normalized by the total number of pixels within the ROI to obtain the microbubble track density (MTD) measure for the ROI.

$$MTD = \frac{\text{number of pixels with microbubbles}}{area\ of\ ROI} \tag{5}$$

This measure is used as a surrogate for active vascular density within the ROI.

Phantom flow model set up and validation

The microbubble detection algorithm was validated on a flow phantom constructed in-house. It consisted of a contrast agent-filled solution in a tank. The flow was generated by a magnetic stirrer, which is placed under the tank. Given that the microbubbles were well mixed, such a setup offers repeatable experimental measurements with different concentrations of microbubbles.

The SonoVue^{TM} (Bracco, Milan) microbubbles were used at six concentrations: 0 µL (control), 0.05µL, 0.1µL, 0.15µL, 0.2µL and 0.25µL, diluted in 0.6L air-saturated water in a tank. A magnetic stirrer was used to stir the solution at 2 rev/second. CEUS data were acquired using the following in vitro scanning protocol. A clinical Philips iU22 ultrasound scanner (linear 3/9MHz broadband linear array transducer, Philips Ultrasound, Bothell, USA) was used to scan the phantom with the following settings: gain = 69%, TGC = manually adjusted, frame rate = 13Hz, compression = 50, persistence = off. The scanner MI was set at 0.06 and the contrast imaging mode on the scanner was used. With the low MI bubble destruction is largely avoided and better reduction of the harmonic component from the tissue is achieved. Three 10-second sequences were obtained for each volume of microbubbles. Analysis of CEUS video sequences was performed offline using software developed in-house, which is written in MATLAB (The Mathworks Inc., Natick, MA, USA). Regions of interest (ROIs) in the middle of the image covering a rectangular area of 245 x 70 pixels (1.75 cm x 0.5 cm) were selected manually. The MTD quantities generated by the proposed method are compared with the known concentrations of the microbubbles. The contrast specific imaging amplitude may be affected by bubble velocity due to signal decorrelation during the pulse sequence but the effect should be small. This is because our approach mainly depends on frequency measurement rather than amplitude, and also given the very short time interval between the two pulses (for a depth of 7.5cm the time internal will be ~0.1ms), the small vessels we are interested where flow is low (much less than 1m/s). Furthermore, the data analysis was performed on video data which is log-compressed, which affects the noise statistics.

In vivo methodology

Five healthy volunteers were recruited from a research centre (Charing Cross Hospital, Imperial College London). The study was approved by the National Research and Ethics Committee (reference 13/LO/0943) and each participant provided written informed consent. CEUS image sequences were acquired on the lower limb with a clinical PHILIPS iU22 scanner (3/9 MHz broadband linear array transducer, Philips Ultrasound, Bothell, USA) with the same settings as *in vitro* experiments. Contrast imaging mode in the scanner is used in this study. All the analyses were performed on such contrast specific images. B mode image is only used for motion estimation. SonoVue^{TM} was diluted using normal saline via a mini-spike system (25mg in 20ml). It was given as a continuous intravenous infusion (VueJect^{TM}, Bracco, Milan) via an 18G cannula sited in an antecubital vein, at a rate of 4.0 mL/min. Subjects were positioned on an examination couch in the left-lateral position, with knees lightly flexed for comfort. Image sampling was taken perpendicular to the skin from the medial head of gastrocnemius in the left leg, and the skin was marked for repeated measures. Care was taken to standardise the relative positions of both subject and imaging clinician using rehearsal. Care was taken to standardise the relative positions of both subject and imaging clinician using rehearsal. Imaging commenced about 10 seconds prior to infusion initiation, and due to the limit of the scanner storage two consecutive acquisitions (~2.5 minutes each) were made to capture the full infusion period of ~5 minutes. Care was taken to minimise image acquisition down-time between recording sessions. Steady-state destruction-reperfusion imaging was conducted approximately 4 minutes after the infusion started, as an existing validated quantification method for comparison. The cannula was flushed with saline and disconnected. Subjects were exercised on a treadmill (walking speed: 2mph, +2%/3-mins, 15-minutes total), and then the imaging studies were repeated. The interval between cessation of exercise was minimised as far as practically possible. Measurements were repeated for each volunteer on consecutive days. One subject was excluded in this study due to acquisition error.

The whole image sequence was divided into five equal image segments of 300 frames each. The last segment was excluded in the data analysis to avoid the end of perfusion. Five region-of-interests were computer generated for the purposes of analysis (dimensions and placement on screen kept constant for all scans; see Figure 4). The MTD for both pre-exercise and after-exercise images were calculated and compared. Repeatability of the proposed method against destruction-reperfusion was also evaluated.

Large blood vessels elimination

Large blood vessels carrying large numbers of microbubbles may distort the measurement of active microvascular density. Visual inspection of scans can identify arteries and veins, and these can be manually removed from the ROI. A comparison before and after manual removal was made.

Non-rigid motion compensation

The motion of lower limb was tracked and corrected before any further processing by an image registration algorithm, MIRT (Myronenko 2006). The algorithm employs a non-rigid motion compensation framework (Lee, et al. 1997, Rueckert, et al. 1999). The MS similarity measure, assuming that Rayleigh speckle noise in consecutive images is correlated, was chosen to deal with noisy B-mode ultrasound images (Myronenko, et al. 2009). Maximum likelihood approach was used to estimate the transformation between the images and hence maximise the conditional probability. To keep the manuscript from being too long, and having the potential radiologist readers in mind, we only included a short description of the algorithm and referred to [M. Andriy, Non-rigid Image Registration: Regularization, Algorithms and Applications,

Ph.D. thesis, Oregon Health & Science University, 2010. http://digitalcommons.ohsu.edu/etd/370/] for details in the manuscript. Here are some details of the method: the registration is done by considering two 2D ultrasound images *I* and *J* acquired at consecutive time instances. The maximum likelihood approach to estimate the transformation *T* between the two images and hence maximise the conditional probability, $p(J(T),| I,T)$, where we assumed that all pixel-wise conditional probabilities are independent and identically distributed. and $J(T)$ denotes the intensity values of pixel after applying the transformation *T*. The *MS* similarity measure assumes that the Rayleigh noise n_1 and n_2 on image *I* and *J* are not independent. If two consecutive images *I* and *J* are taken with sufficiently high frame rate, which is the case for modern ultrasound devices, the speckle noise formation between the consecutive frames is similar, and the noise n_1 and n_2 are correlated. Then, the conditional probability becomes:

$$p(J_n(T)|I_n,T) = \frac{2(1-\rho)\eta^2}{D(1+\eta^2)^2}\left(1-\frac{4\rho\eta^2}{(1+\eta^2)^2}\right)^{-\frac{3}{2}} \quad (6)$$

where *D* is the scaling constant of the dynamic range, ρ is the correlation coefficient and $\eta = n_1/n_2$

The registration and correction were firstly conducted on the simultaneously acquired B-mode sequence and then transferred to CEUS image sequence.

Destruction and Replenishment (DR) analysis

The *in vivo* flow quantification was calculated using destruction replenishment time-intensity data (Lindner, et al. 2008, Wei, et al. 1998). A frame obtained 0.08 second after destruction is used as the background and is subtracted from subsequent frames to eliminate signal from non-

capillary vessels (Belcik, et al. 2015). The replenishment curve was fitted with a mono exponential function, $y = A(1 - e^{-\beta t})$, where y is video intensity, A is plateau intensity and β is the rate constant using a non-linear least squares fitting algorithm in MATLAB. The time sequence analysed was measured from destruction flash to the end of the following 500 frames. Peak intensity (A), blood flow ($A \times \beta$) and flow reserve (ratio of blood flow after exercise to resting blood flow) were calculated from this model, and compared with the results obtained by our microbubble detection algorithm.

Statistical analysis

The microbubble track density (MTD) measures were calculated and the difference before and after exercise tested using paired samples *t* tests. A two-tailed test was used, with alpha set at 0.05. Statistical analysis was performed using online GraphPad Prism 6 (GraphPad Software Inc., San Diego, California, USA). For reproducibility the intra-class correlation coefficients (ICC) of MTD and DR methods for the four subjects' two repeats on different days were calculated and compared.

Results

Phantom validation

By examining the HLFR histograms two distinct peaks were detected at HLFR = 0.2 ($\widehat{f'}$= 0.31 rad/s, microbubbles) and HLFR = 0.75 ($\widehat{f'}$= 0.31 rad/s, noise). The locations of both peaks were similar for different concentrations of microbubbles. The segmentation results of the phantom with five microbubble concentrations are shown in Figure 5. It can be seen that more microbubbles were detected at higher concentration.

The linear relationship between MTD and concentration is illustrated in Figure 6, with an R-square value of 0.89.

In vivo results

The plots of normalised HLFR of four subjects before and after exercise are displayed in Figure 3. Two distinct peaks are seen, the lower one corresponding to microbubbles and the higher peak for noise. The thresholds T_{HLFR} of the four subjects were automatically determined according to that described in section Microbubble detection algorithm to be 0.12 ($\widehat{f'}$= 2.51, before exercise) and 0.167 ($\widehat{f'}$= 2.51, after exercise) for subject 1, 0.093 ($\widehat{f'}$= 1.88, before exercise) and 0.14 ($\widehat{f'}$= 1.88, after exercise) for subject 2, 0.16 ($\widehat{f'}$= 2.51, before exercise) and 0.15 ($\widehat{f'}$= 1.88, after exercise) for subject 3, and 0.4 ($\widehat{f'}$= 1.26, before exercise) and 0.35 ($\widehat{f'}$= 0.94, after exercise) for subject 4, and the unit of the frequency f'is rad/s.

The segmentation results of four subjects before and after exercise are provided in Figure 7. It can be seen that the segmented microbubble areas increased after exercise.

Destruction and Replenishment analysis

The time intensity curves, fitted with the mono exponential function before and after exercise with a repeated scan are shown in Figure 4. The perfusion was increased after exercise.

364 *Reproducibility*

365 The percentage change of microbubble track density after exercise for each scan is compared
366 (Figure 8). The average percentage increase of microbubble track density (mean ± SD) was
367 138.2% ± 79.8 at the first day and 119.4% ± 62.7 at the second day, and the average percentage
368 increase of MTD for two days was 128.8%. While for the existing DR method, the average
369 percentage increase of peak intensity (mean ± SD) was 75.6% ± 71.6 at the first day and 234.7%
370 ± 169.3 at the second day, and the average percentage increase of peak intensity for two days
371 was 155.1%. For DR blood flow measurement, the average percentage increase (mean ± SD)
372 was 213.8% ± 191.3 at the first day and 341.1% ± 215.4 at the second day for DR analysis, and
373 the average percentage increase for two days was 277.4%. Furthermore, the DR average flow
374 reserve (mean ± SD) was 3.1 ± 1.9 at the first day and 4.4 ± 2.2 at the second day for DR
375 analysis, and the average flow reserve for two days was 3.7. Figure 9 also shows using a scatter
376 plot how repeatable each method is. The proposed approach demonstrated excellent agreement
377 on repeated measurements with high reproducibility (ICC = 0.96, $p = 0.008$), while the existing
378 state-of-art DR analysis showed poor reproducibility of peak intensity (ICC = -0.39, $p = 0.61$),
379 and better reproducibility of blood flow (ICC = 0.78, $p = 0.09$) and flow reserve (ICC = 0.78,
380 $p = 0.09$).

381

382 *Analysis with large blood vessels*

383 The MTD using the proposed algorithm without removing large vessel signals was also
384 calculated. The average percentage increase of microbubble track density (mean ± SD) was
385 130.2% ± 69.8 at the first day and 115.8% ± 63.3 at the second day. The average percentage
386 increase of MTD was 123% for two days. Only a small change of ~6% in the averaged data is

found when comparing to the results with large vessels removed. The inclusion of large vessels does not change the repeatability of the results either (ICC = 0.97 vs. 0.96).

Discussion

A temporal and spatial image analysis method has been developed for detection and segmentation of microbubble signals to generate MTD, a quantitative surrogate measure for vascular density /tissue active capillary density. The method was validated in-vitro and then applied to healthy lower limb CEUS images to quantify active vascular density. The in-vitro results show an excellent linear relationship between the microbubble concentration and the MDT measure. The in vivo data on human lower limbs show a significant increase in MDT measure after exercise (129%) and the results are highly repeatable (ICC=0.96).

It should be noted that the bubble velocity variations could affect the frequency spectrum of the temporal pixel signal. However, given the two very different types of signals we want to separate, slow microbubble movement in microvasculature versus very high frequency noise, and only a threshold (of high to low frequency ratio) is required, there is certain room in the methods to accommodate velocity variations.

Quantification of active vascular density is valuable in a wide range of clinical applications. While CEUS imaging is increasingly used in clinical imaging and research, its repeatability and accuracy are still poor, largely due to the various factors that affect the image intensity-based quantification measures (Tang, et al. 2011). Since the relative frequency feature used in this method is image intensity independent and is robust to the various confounding factors

such as attenuation, the approach may potentially offer reliable and repeatable quantification results. It is shown that our method is much more repeatable than the accepted disruption-replenishment analysis which is image intensity based (ICC of 0.96 vs 0.78). Moreover, our approach can also deal with microbubbles travelling perpendicular to the 2D imaging plane.

Also, it is assumed that the concentration of detectable microbubbles is relatively low, given the typical clinical dose and the fact that many bubbles injected will be invisible under the clinical high frequency.

Besides reproducibility, this proposed method can also help address another key issue of the existing method for limb vascular density quantification; the low SNR associated with low basal blood flows in humans. As the proposed technique makes use of temporal information accumulated over a couple of minutes, it is more robust to noise. The in vivo data of this study demonstrated detection of a significant amount of bubble signal corresponding to basal blood flow.

The proposed method is based on temporal analysis of individual pixels so it is sensitive to the motion effect. As the motion of lower limb can be non-rigid (muscle movement may result in the images changing shape, and these shape changes cannot be corrected by a rigid body transformation), we employed a non-rigid motion correction (Myronenko, et al. 2009) to reduce motion artefacts. While this correction technique seems to be effective in correcting motion and allows the generation of repeatable quantification results, any remaining motion that is

uncorrected for could potentially introduce a bias in the quantification by magnifying vessel footprints.

For some applications the primary target of interest is small capillary vessels, and hence the existence of large vessels, e.g. in the lower limb images in this study, is less desirable and may affect the quantification result. In this study we have identified the apparent large vessels in the data by visual inspections and manually removed them. We then compared the quantification results with or without the large vessels. In this case the results with and without large vessels in this case are very similar. The average percentage increase of MTD with large vessels is slightly smaller (123%) than the one without large vessels (129%) and the difference of average percentage increase is not statistically significant. This indicates that the result is not significantly affected by large vessels. As our approach counts the areas that any microbubble covers in CEUS images, even a single bubble slowly flowing through a small vessel would cover a significant area due to the point spread function of imaging system being much larger than the size of a microbubble/capillary. Therefore our approach seems to favour small vessels than larger ones which might explain why the existence of large vessels did not have a significant effect.

Our destruction reperfusion analysis is concordant with that reported in existing literature (Lindner, et al. 2008). The peak intensity and blood flow measurements increased after exercise. However, the reproducibility is not reported in that study. When our DR method is compared with MTD, the reproducibility characteristics of MTD are much more favourable.

The delay between the exercise and the imaging (~2 minutes) could reduce the flow reserve measurements. Another factor is that the physical exercise in this study is not very stressful so only a minor vasodilatation is expected. Both factors contributed to the low flow reserve comparing to that in (Lindner, et al. 2008). It should be noted that comparing to perfusion, the vascular density / MTD measured in this study is less dependent on e.g. the applied stress (exercise), the subject's physical condition, and the time taken from exercise to imaging.

It should be noted that our approach is different to the maximum intensity projection (MIP) (Anderson, et al. 1990, Parker, et al. 1988), which display the maximum image intensity during the whole acquisition period at each pixel. While MIP is a good tool to visualise vascular morphology, it is still image intensity based and has similar issues as other existing techniques when used for vascular density quantification.

The proposed method can be affected by the concentration of bubbles. Using too high a concentration of bubbles may cause saturation in the bubble detection results. Such saturation can be dealt with by either taking shorter video sequences, or by applying a statistical formula (Siepmann, et al. 2010). Furthermore, it should be noted that the number of subjects is low (n=4) in this study and the proof-of-concept nature of this study. Further work on more subjects would be useful to confirm the robustness of our method.

The present study does not measure kinetics and hence perfusion. However, the frequency features of the image sequence data have information that allows not only an effective

separation of bubbles from noise, but also potentially the velocity information of the blood flow. A pixel within a vessel with faster flow will generate higher temporal frequencies due to the more frequent appearance of microbubbles in the pixel temporal signal. These frequencies will also be dependent on microbubble concentration and further studies should be conducted to explore this extra information in the CEUS temporal signals.

It should be noted that the out-of-plane motion could still affect the quantification, if homogeneity in microvessel distribution in the tissue cannot be assumed. Further studies to take into account out-of-plane motion, techniques such as 3D US/CEUS imaging and motion correction could potentially improve the quantification results.

We observed that there is often single-pixel noise remaining, known as salt-and-pepper noise, after our bubble detection algorithm. To remove such noise but keep microbubble signals, we used a 3x3 median filter. The size of the filter is determined by measuring apparent microbubble sizes at various image depths under the experimental system settings described in the Methods section. The smallest bubble size is ~ 5 by 5 pixels. The image resolution is ~ 14 pixel per mm so the pixel size is ~ 0.07mm

This technique has great potential for clinical translation. Practically, it would be feasible to use tens of seconds of standard clinical CEUS scan data during plateau phase, and the quantification process can be fully automated with high repeatability. It has great potential in the real-time assessment of limb vascular density /active capillary density in patients with peripheral vascular density deficits. The need for cardiovascular ionotropic support can have

negative effects on peripheral vascular density, and CEUS may be able to guide intravascular filling needs and ionotropic support. CEUS could be used to accurately quantify capillary vascular density in post-operative surgical flap monitoring, guiding patient management and decision making. Our automated CEUS method could be used in an outpatient setting, provide a potentially valuable biomarker for clinically significant peripheral arterial disease, or attribute information to the management of the patient with a diabetic foot (prognosis, surgical planning, treatment monitoring). It also has the potential to be extended to other clinical applications, e.g. quantification of carotid/aortic plaque neovascularisation, breast screening or cancer monitoring.

Conclusions

The proposed microbubble detection method demonstrated excellent accuracy and repeatability in quantifying active vascular density and has great potential for clinical translation in the assessment of lower limb vascular density and beyond.

References

Ackermann D, Schmitz G. Detection and Tracking of Multiple Microbubbles in Ultrasound B-Mode Images. IEEE Trans Ultrason Ferroelectr Freq Control 2016; 63:72-82.

Amarteifio E, Krix M, Wormsbecher S, Demirel S, Braun S, Delorme S, Kauczor HU, Bockler D, Weber MA. Dynamic contrast-enhanced ultrasound for assessment of therapy effects on skeletal muscle microcirculation in peripheral arterial disease: pilot study. Eur J Radiol 2013; 82:640-6.

Amarteifio E, Weber MA, Wormsbecher S, Demirel S, Krakowski-Roosen H, Jores A, Braun S, Delorme S, Bockler D, Kauczor HU, Krix M. Dynamic contrast-enhanced ultrasound for assessment of skeletal muscle microcirculation in peripheral arterial disease. Invest Radiol 2011; 46:504-8.

Anderson CM, Saloner D, Tsuruda JS, Shapeero LG, Lee RE. Artifacts in maximum-intensity-projection display of MR angiograms. AJR. American journal of roentgenology 1990; 154:623-9.

Bar-Zion AD, Tremblay-Darveau C, Yin M, Adam D, Foster FS. Denoising of Contrast-Enhanced Ultrasound Cine Sequences Based on a Multiplicative Model. IEEE transactions on bio-medical engineering 2015; 62:1969-80.

Barrois G, Coron A, Payen T, Dizeux A, Bridal L. A multiplicative model for improving microvascular flow estimation in dynamic contrast-enhanced ultrasound (DCE-US): theory and experimental validation. IEEE Trans Ultrason Ferroelectr Freq Control 2013; 60:2284-94.

Belcik JT, Davidson BP, Foster T, Qi Y, Zhao Y, Peters D, Lindner JR. Contrast-enhanced ultrasound assessment of impaired adipose tissue and muscle perfusion in insulin-resistant mice. Circ Cardiovasc Imaging 2015; 8.

Cheung WK, Gujral DM, Shah BN, Chahal NS, Bhattacharyya S, Cosgrove DO, Eckersley RJ, Harrington KJ, Senior R, Nutting CM, Tang MX. Attenuation Correction and Normalisation for Quantification of Contrast Enhancement in Ultrasound Images of Carotid Arteries. Ultrasound Med Biol 2015; 41:1876-83.

Christensen-Jeffries K, Browning RJ, Tang MX, Dunsby C, Eckersley RJ. In Vivo Acoustic Super-Resolution and Super-Resolved Velocity Mapping Using Microbubbles. Ieee T Med Imaging 2015; 34:433-40.

Claudon M, Dietrich CF, Choi BI, Cosgrove DO, Kudo M, Nolsoe CP, Piscaglia F, Wilson SR, Barr RG, Chammas MC, Chaubal NG, Chen MH, Clevert DA, Correas JM, Ding H, Forsberg F, Fowlkes JB, Gibson RN, Goldberg BB, Lassau N, Leen EL, Mattrey RF, Moriyasu F, Solbiati L, Weskott HP, Xu HX, World Federation for Ultrasound in M, European Federation of Societies for U. Guidelines and good clinical practice recommendations for Contrast Enhanced Ultrasound (CEUS) in the liver - update 2012: A WFUMB-EFSUMB initiative in cooperation with representatives of AFSUMB, AIUM, ASUM, FLAUS and ICUS. Ultrasound Med Biol 2013; 39:187-210.

Duerschmied D, Zhou Q, Rink E, Harder D, Freund G, Olschewski M, Bode C, Hehrlein C. Simplified contrast ultrasound accurately reveals muscle perfusion deficits and reflects collateralization in PAD. Atherosclerosis 2009; 202:505-12.

Errico CE, Pierre J, Pezet S, Desailly Y, Lenkei Z, Couture O, Tanter M. Ultrafast ultrasound localization microscopy for deep super-resolution vascular imaging. Nature 2015; 527:499-+.

Hellings WE, Peeters W, Moll FL, Piers SRD, van Setten J, Van der Spek PJ, de Vries JPPM, Seldenrijk KA, De Bruin PC, Vink A, Velema E, de Kleijn DPV, Pasterkamp G. Composition of Carotid Atherosclerotic Plaque Is Associated With Cardiovascular Outcome A Prognostic Study. Circulation 2010; 121:1941-U111.

Hoogi A, Akkus Z, van den Oord SC, ten Kate GL, Schinkel AF, Bosch JG, de Jong N, Adam D, van der Steen AF. Quantitative analysis of ultrasound contrast flow behavior in carotid plaque neovasculature. Ultrasound Med Biol 2012; 38:2072-83.

Huang PT, Huang FG, Zou CP, Sun HY, Tian XQ, Yang Y, Tang JF, Yang PL, Wang XT. Contrast-enhanced sonographic characteristics of neovascularization in carotid atherosclerotic plaques. Journal of clinical ultrasound : JCU 2008; 36:346-51.

Krix M, Krakowski-Roosen H, Amarteifio E, Furstenberger S, Delorme S, Kauczor HU, Weber MA. Comparison of transient arterial occlusion and muscle exercise provocation for assessment of perfusion reserve in skeletal muscle with real-time contrast-enhanced ultrasound. Eur J Radiol 2011; 78:419-24.

Krix M, Krakowski-Roosen H, Kauczor HU, Delorme S, Weber MA. Real-time contrast-enhanced ultrasound for the assessment of perfusion dynamics in skeletal muscle. Ultrasound Med Biol 2009; 35:1587-95.

Lee S, Wolberg G, Shin SY. Scattered data interpolation with multilevel B-splines. Ieee T Vis Comput Gr 1997; 3:228-44.

Lindner JR, Womack L, Barrett EJ, Weltman J, Price W, Harthun NL, Kaul S, Patrie JT. Limb stress-rest perfusion imaging with contrast ultrasound for the assessment of peripheral arterial disease severity. JACC Cardiovasc Imaging 2008; 1:343-50.

Mitchell WK, Phillips BE, Williams JP, Rankin D, Smith K, Lund JN, Atherton PJ. Development of a new Sonovue contrast-enhanced ultrasound approach reveals temporal and age-related features of muscle microvascular responses to feeding. Physiol Rep 2013; 1:e00119.

583 Myronenko A. Non-rigid Image Registration: Regularization,Algorithms and Applications. Ph.D. thesis,
584 Oregon Health & Science University 2006.
585 Myronenko A, Song X, Sahn DJ. Maximum Likelihood Motion Estimation in 3D Echocardiography
586 through Non-rigid Registration in Spherical Coordinates. Functional Imaging and Modeling of
587 the Heart, Lecture Notes in Computer Science 2009; 5528:427-36.
588 Otsu N. A Threshold Selection Method from Gray-Level Histograms. IEEE Transactions on Systems,
589 Man and Cybernetics 1979; 9:62-66.
590 Parker DL, Wu J, van Bree RE. 1988 Three-dimensional vascular reconstruction from projections: a
591 theoretical review. *Engineering in Medicine and Biology Society, 1988. Proceedings of the*
592 *Annual International Conference of the IEEE*, 399-400 vol.1.
593 Rueckert D, Sonoda LI, Hayes C, Hill DLG, Leach MO, Hawkes DJ. Nonrigid registration using free-form
594 deformations: Application to breast MR images. Ieee T Med Imaging 1999; 18:712-21.
595 Senior R, Moreo A, Gaibazzi N, Agati L, Tiemann K, Shivalkar B, von Bardeleben S, Galiuto L, Lardoux
596 H, Trocino G, Carrio I, Le Guludec D, Sambuceti G, Becher H, Colonna P, Ten Cate F, Bramucci
597 E, Cohen A, Bezante G, Aggeli C, Kasprzak JD. Comparison of sulfur hexafluoride microbubble
598 (SonoVue)-enhanced myocardial contrast echocardiography with gated single-photon
599 emission computed tomography for detection of significant coronary artery disease: a large
600 European multicenter study. J Am Coll Cardiol 2013; 62:1353-61.
601 Siepmann M, Reinhardt M, Schmitz G. A statistical model for the quantification of microbubbles in
602 destructive imaging. Invest Radiol 2010; 45:592-9.
603 Song Y, Li Y, Wang PJ, Gao Y. Contrast-enhanced ultrasonography of skeletal muscles for type 2
604 diabetes mellitus patients with microvascular complications. International journal of clinical
605 and experimental medicine 2014; 7:573-9.
606 Tang MX, Eckersley RJ. Frequency and pressure dependent attenuation and scattering by
607 microbubbles. Ultrasound Med Biol 2007; 33:164-8.
608 Tang MX, Mulvana H, Gauthier T, Lim AKP, Cosgrove DO, Eckersley RJ, Stride E. Quantitative contrast-
609 enhanced ultrasound imaging: a review of sources of variability. Interface Focus 2011; 1:520-
610 39.
611 Viessmann OM, Eckersley RJ, Christensen-Jeffries K, Tang MX, Dunsby C. Acoustic super-resolution
612 with ultrasound and microbubbles. Phys Med Biol 2013; 58:6447-58.
613 Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S. Quantification of myocardial blood flow
614 with ultrasound-induced destruction of microbubbles administered as a constant venous
615 infusion. Circulation 1998; 97:473-83.
616 Xiong L, Deng YB, Zhu Y, Liu YN, Bi XJ. Correlation of Carotid Plaque Neovascularization Detected by
617 Using Contrast-enhanced US with Clinical Symptoms. Radiology 2009; 251:583-89.
618 Yildiz YO, Eckersley RJ, Senior R, Lim AK, Cosgrove D, Tang MX. Correction of Non-Linear Propagation
619 Artifact in Contrast-Enhanced Ultrasound Imaging of Carotid Arteries: Methods and in Vitro
620 Evaluation. Ultrasound Med Biol 2015; 41:1938-47.

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622 Figure 1: The time intensity curve and power spectrum of (A, B) microbubbles (C, D) noise

623 The power spectrum density in Fig2B and 2D represents the frequency composition of the pixel
624 temporal signal (Fig2A and 2C). Such frequency spectrum indicates how fast the pixel signal
625 changes over time. For a pixel containing only noise, the signal changes has both fast (high
626 frequency) and slow (low frequency) components in the spectrum (broadband) (Fig2D). For

bubble signal the change is slow and it has a peak at lower frequencies in the spectrum (Fig2B). The position of the peak depends on the bubble flow velocity as explained in the Methods section and equation (2).

Figure 2: Contrast-enhanced ultrasound screen captures from human gastrocnemius muscle in vivo, after injection of Sonovue. (A) CEUS mode image with tissue and microbubble signals are labelled with arrows. and (B) B-mode image

Figure 3: The normalised HLFR of (A, B) subject 1, (C, D) subject 2, (E, F) subject 3, (G, H) subject 4 before and after exercise of first day scan

Figure 4: The disruption-replenishment time intensity curves with mono exponential of (A, B) subject 1, (C, D) subject 2, (E, F) subject 3, (G, H) subject 4 for the first and second day scans [BE - before exercise, AE - after exercise]

Figure 5: The segmentation results of the phantom with five microbubbles concentrations (A) 0.05µL (B) 0.1µL (C) 0.15µL (D) 0.2µL (E) 0.25µL [The height of the ROI: 0.7cm]

Figure 6: Microbubble track density measure versus microbubble concentration in the phantom. Three repeats of washing and re-injecting bubbles (Wash) and three repeats for each bubble injection were made

Figure 7: The CEUS segmentation results of subject 1 (A, B), subject 2 (C, D), subject 3 (E, F) and subject 4 (G, H), taken from gastrocnemius before and after exercise

Figure 8: (A) Microbubble track density quantification by our microbubble detection algorithm per scan and (B) Peak intensity, (C) Blood flow, (D) Flow reserve by Destruction and Replenishment analysis before and after exercise

Figure 9: The plot of percentage change of first day scan vs second day scan by (A) Microbubble track density quantification and (B) Peak intensity, (C) Blood flow, (D) Flow reserve by destruction-replenishment analysis. (n=4)

Video: Contrast-enhanced ultrasound movie from human gastrocnemius muscle in vivo, after injection of Sonovue. (left panel) CEUS mode image with tissue and microbubble signals are labelled with arrows and (right panel) B-mode image