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Transcutaneous very-high-resolution ultrasound to quantify arterial wall layers of muscular and elastic arteries: Validation of a method

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ABSTRACT

Background: High-resolution ultrasound (HRU) is used to measure carotid intima-media thickness (IMT). We postulated that very-high-resolution ultrasound (VHRU, 25–55 MHz) provides more detailed information on arterial morphology.
Methods: Rabbit and pig arterial specimens and artificial elastin membranes were studied with HRU and VHRU, and compared to histology. Bilateral carotid, brachial, radial, ulnar, femoral, and tibial arteries were imaged in vivo in 15 humans to determine the precision of VHRU and in 53 teenagers to compare VHRU to HRU.
Results: The assessment of IMT, adventitia thickness (AT) and combined intima-media-adventitia thickness (IMAT) in muscular arteries was accurate and precise by VHRU with the exception that the AT of the smallest arteries was not delineated with 25 MHz. VHRU was accurate and precise for IMAT in small and for IMT in large elastic arteries and allowed to qualitatively assess elastin fibers of the media. HRU was accurate for IMT of large muscular and elastic arteries only. Intima thickness (IT) was grossly overestimated by both VHRU and HRU.
Conclusion: Transcutaneous VHRU provides a noninvasive method of quantifying elastic and muscular arterial AT, IMT and IMAT in children and adults, but neither VHRU nor HRU is able to assess IT in non-diseased vessels.

1. Introduction

The noninvasive measurement of the combined intima-media thickness (IMT) by conventional high-resolution B-mode vascular ultrasound imaging (HRU) with transducers in the 5–15 MHz range has become widely used as a surrogate marker for the progression of atherosclerotic disease [1–4]. HRU has been validated for the carotid [5–8] and femoral arteries [9] and applied in a wide range of clinical settings both in adults and adolescents [10,11]. Nevertheless, the spatial resolution of HRU systems appears insufficient for more detailed imaging of different arterial wall layers and for the examination of the peripheral vasculature and the carotid arteries of the younger child.

The recent emergence of commercially available very-high frequency ultrasound systems (VHRU) with transducers in the 25–55 MHz range allows imaging of more superficial tissues in almost microscopic detail. VHRU is, however, precluded in the imaging of more deep structures due to attenuation of the ultrasound beam. To date, VHRU has mainly been used to image coronary arteries by intravascular ultrasound (IVUS [12]), for small animal research [13] and, most recently, to evaluate the morphology of peripheral arteries [14,15]. According to these studies it was possible to accurately determine media and total wall thickness in superficial arteries of pigs [14]. Furthermore, the measurement of the intima thickness (IT) separately from the media was validated against silicone phantoms and vascular specimens in the IT range of 0.15–0.40 mm. This method was then applied to study peripheral muscular arteries in healthy volunteers in vivo, but their measurements appeared to be out of the physiologic range [15]. To the best of our knowledge, the utility of VHRU has not been examined systematically, in terms of varying transducer frequencies, and their ability to measure the intima, media and adventitia separately and together in muscular and elastic arteries in adults and children.

The primary aims of this study were therefore to investigate the accuracy and precision of VHRU in the assessment of the intima, media and adventitia in small and large muscular and elastic arteries as well as to compare VHRU with HRU.

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2. Methods

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Research Ethics Board. The use of VHRU in human subjects was in addition approved by Health Canada. Study participation required written informed consent by all human participants.

2.1. Accuracy measurements

To determine the accuracy of VHRU-derived vascular wall measurements, 1–2 cm-long specimens of the common carotid, proximal brachial, renal, and common femoral arteries were obtained within 2 h post-mortem from 12 rabbits (weight 3–4 kg) and 7 pigs (weight about 35 kg) in order to obtain a range of different vessel structures and sizes. Aortic specimens were also obtained from the rabbits. The periadventitial tissue was removed from large pig specimens. All specimens were fixed in buffered [1,10] formalin for at least 24 h. The effect of formalin fixation on layer dimensions was tested in a subset of specimens.

The fixed specimen was placed on a Petri dish, imbedded in ultrasonographic gel, and imaged in long and short axis sweeps using the commercially available Vevo 770 ultrasound system (Visualsonics, Toronto, Canada) with mechanical 25, 35, 40 and 55 MHz linear transducers. Depth range, focal depth and pixel resolution (distance/pixel) of ultrasound images were: 1–8 mm, 4.5 mm, and 0.0156 mm/pixel for the 55 MHz transducer (RMV708); 1.5–10 mm, 6.0 mm, and 0.0196 mm/pixel for the 40 MHz transducer (RMV704); 4.5–13 mm, 9.0 mm, and 0.0196 mm/pixel for the 35 MHz transducer (RMV712); and 6.5–22.5 mm, 15 mm, and 0.0357 mm/pixel for the 25 MHz (RMV710B) transducer. Larger pig arteries were also imaged by conventional HRU using the Vivid 7 ultrasound system (GE Medical Systems, Horten, Norway) with a linear 12 MHz transducer. High-quality long-axis still images from the arterial far wall at the largest vascular diameter and with good distinction between interfaces were used for measurements of intima thickness (IT), intima-media thickness (IMT), and intima-media-adventitia thickness (IMAT). IT was defined as the distance from the lumen-vessel interface to the interface between the internal elastic lamina and the media, IMT as the distance from the lumen-vessel interface to the interface between the media and the external elastic lamina, and IMAT as the distance from the lumen-vessel interface to the interface between the adventitia and the surrounding tissue. Ultrasound thickness was defined, based on artificial elastic membrane appearance and vessel appearance on histology, as the distance between leading edges of interfacing surfaces excluding technical scatter above and below the internal and external elastic lamina [16]. Gain settings were optimized in order to minimize the amount of scatter of the elastic laminas and to produce a sharp distinction between the different layers. Off-line measurements of VHRU images were performed manually by a single investigator (TS) with electronic calipers in a very-high-resolution zoom window using the Vevo 770 software (version 2.3.0). The IMT obtained by HRU was measured with Carotid Analyzer for Research (Version 5.7.4, Medical Imaging Applications LLC). IT and IMAT were measured with a manual caliper using the EchoPAC PC (Version 7.1.2, GE Vingmed Ultrasound). Media thickness (MT) was calculated as the difference between IMT and IT, and adventitia thickness (AT) as the difference between IMAT and IMT. Three measurements were obtained, averaged, and used in subsequent analyses. The gel was removed and the specimen replaced in the formalin fixative prior to histologic analysis.

2.2. Histological analysis

Each specimen was cut in cross section, embedded in paraffin, sectioned, and stained with Elastic (Verhoeff's) Trichrome. Stained glasses were analyzed using a Leica EZ4D stereomicroscope (Spectronic Analytical Instruments) with a 20 or 30 times magnification. A standard ruler was used for calibration. Measurements were performed off-line from stored images with the Leica statistical software. Anatomical orientation of the vessel was optimized and matched to the corresponding long and short axis ultrasound image. The thickness of IT, IMT and IMAT were measured and MT and AT calculated as described above. The histological analyses were made independently, at least one week after the ultrasound measurements in order to blind the single observer. The mean of 3 measurements was used in subsequent analyses.

2.3. Artificial elastin membranes

Recombinant elastin-like polypeptide (EP) based membranes with a standard area of approximately one square centimeter and weights between 0.25 and 3 mg were produced from EP4 peptides as previously described [17]. The membranes were immersed in water in a Petri dish and scanned with the different VHRU-transducers. Membrane thickness was measured off-line with the investigator blinded of the true thickness. The thickness of the membrane in the ultrasound image was defined similarly to the IT as the distance between the leading and the trailing edges of the echo reflection excluding scatter. The membranes were then embedded in agar, turned on the side, embedded in paraffin, cut and sectioned, and the true thickness determined by light microscopy as described above.

2.4. Image acquisition in vivo

Fifteen healthy participants between 4 and 45 years of life were enrolled to examine the precision and reproducibility of VHRU of various arteries as described below, using 25, 35 and 55 MHz transducers. In addition, the common carotid artery was imaged with the 12, 25 and 35 MHz transducers in 53 healthy adolescents (age 14.7 ± 1.6 years, range 12.3–17.8) to compare VHRU with HRU.

Ultrasound recordings were obtained from the corresponding right- and left-sided arterial locations in a supine position from resting, non-sedated participants and stored for off-line analysis. Care was taken not to compress the arteries during image acquisition. The radial and ulnar arteries were studied 1–2 cm proximal to the skin fold that separates the palma manus from the anterior antebrachial region, the dorsal pedal arteries proximal to the first metatarsal bone, the dorsal tibial arteries at the medial malleolar level, the common carotid artery approximately 1 cm proximal to the carotid bulb, the brachial artery approximately 2 cm proximal to the cubital skin fold, and the common femoral artery at the inguinal skin fold.

Off-line analysis was performed on high-quality vascular images, showing a good distinction between structural interfaces. End-diastolic lumen diameters were obtained with reference to the simultaneously recorded electrocardiogram. IT, IMT, IMAT were measured from the far wall with the leading edge technique during end-diastole. The trailing edge technique was applied for the near-wall of the carotid artery using the 35 MHz transducer due to limited penetration. MT and AT was calculated as described above.

Intraobserver variability was assessed from image clips by one investigator (TS) on two separate occasions. Interobserver variability was assessed from the same image clips by an experienced vascular-imaging technician. Reproducibility (test-retest variability) was assessed by one investigator (TS) from image clips obtained on two separate occasions.

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The precision of the common carotid IMT imaged with the 12 MHz transducer has recently been assessed in conjunction with a quality assurance program in our laboratory ($N = 16$). The intraobserver agreement displayed a mean difference −0.001 mm, proportion of the mean difference −0.3%, and 95% limits of agreement −0.02 to 0.02 mm (CV 2%), the interobserver agreement a mean difference −0.01 mm, proportion of the mean difference −1.8%, and 95% limits of agreement −0.08 to 0.06 mm (CV 7%), and the test-retest agreement a mean difference −0.003, proportion of the mean difference −0.5%, and 95% limits of agreement −0.08 to 0.07 mm (CV 8%).

2.5. Statistics

Results are reported as mean with SD, ratio, and range as appropriate. The relationship between two variables was assessed from bivariate scatter plots and the Pearson correlation coefficient calculated. Accuracy agreement was assessed by Bland–Altman plots which were constructed using the formula X = (histological thickness + ultrasound thickness)/2 and Y = histological thickness − ultrasound thickness [18]. Precision agreement was assessed using the same formula. Agreement was quantified by calculating mean differences, the proportion of the mean (%), and 95% limits of agreement. Right and left arterial interrogation sites were included separately in the agreement analyses. Paired Student t-test was used for statistical comparisons. Statistical analyzes were performed with Prism 5 (GraphPad Software, San Diego, California).

3. Results

3.1. Effect of formalin fixation and storage

A set of arterial specimens ($n = 9$) was imaged with the 55 MHz transducer before and after 24 h of fixation. While formalin fixation resulted in no obvious change in the qualitative appearance of the vessel, mild shrinkage of 3.2% and 3.7% was observed for IMT and IMAT, respectively (p = ns). Another set of vessel specimens ($n = 17$) was imaged with the 55 MHz transducer two and 26 days, respectively, after formalin fixation, and again no significant change in the qualitative appearance was observed while the mean shrinkage was 1.7% for IMT and 1.6% for IMAT (p = ns).

3.2. Artificial elastin membranes

A good correlation was found ($r = 0.76$, $p < 0.001$, $n = 16$) for membrane thickness between microscopy (range 0.012–0.038 mm) and 55-MHz-derived measurements. However, the ultrasound thickness exceeded the microscopically determined thickness on average by 0.024 mm (95% limits of agreement 0.011–0.037 mm) with the 55 MHz, by 0.041 mm (95% LOA 0.016–0.067 mm) with the 40 MHz, by 0.048 mm (95% LOA 0.032–0.063 mm) with the 35 MHz, and by 0.090 mm (95% LOA 0.031–0.148 mm) with the 25 MHz transducer, respectively. An image of the membrane is displayed in Fig. 1A.

3.3. Qualitative appearance of vessel specimens in comparison to histology

The appearances of the artificial elastin membrane on ultrasound and the artery on histology were used to identify the internal elastic lamina, tunica media, external elastic lamina, tunica adventitia and the external vessel wall border in the ultrasound image (Fig. 1). We were unable to identify any other subtle structures of the intima layer than the internal elastic lamina. The muscular artery specimens displayed a triple line pattern and the elastic artery specimens a double line pattern in the ultrasound image (Fig. 1). The tunica media of the elastic arteries appeared qualitatively more echogenic when compared with the muscular arteries, a finding consistent with the elastic fibers present in the media on histology (Fig. 1). This finding was most obvious on the recordings with 35–55 MHz. The media of elastic arteries appeared more echoluscent with 25 MHz and 12 MHz. As a consequence, the media to adventitia interface was best identified by lower frequency imaging and less well discernable by highest frequency recordings. Furthermore, the limitations in the resolution of the 12 and 25 MHz transducers precluded the imaging of the wall in the smallest arteries. As a result, we were unable to delineate the external elastic lamina in the small rabbit elastic arteries with 25–55 MHz or image the adventitia of the smallest muscular arteries using the 12 or 25 MHz transducers due to fusion of the external elastic lamina and the external vessel wall border in the image.

3.4. Accuracy of in vitro thickness measurements

Table 1 displays the accuracy of VHRU measurements of arterial rabbit specimens. Rabbits were comparable in weight (3–4 kg) to a full-term human neonate. Good agreement was observed for IMT, AT, and IMAT between the 55 MHz and the histological findings of muscular arteries (Fig. 2A–C). The external elastic lamina was not discernable in elastic arteries, precluding the accurate evaluation of IMT and AT in the rabbits. Moreover, the agreement between 55 MHz-derived measurements of muscular and elastic arterial IT and the corresponding histological findings was poor (Fig. 2G). IT was significantly overestimated and consequently the calculated MT was significantly underestimated.

Limitations in the resolution of the 12 MHz transducer precluded the accuracy assessment of HRU for the small rabbit vessels. Thus, the accuracy of conventional HRU in comparison to VHRU could only be tested in larger pigs, which were comparable in weight (35 kg) to healthy 8–14-year-old children [19]. Good agreement between ultrasound and histological IMT of muscular and elastic pig arteries was observed (Fig. 2A and D, Table 2). The agreement for AT and IMAT was good for muscular arteries using 35, 40 and 55 MHz transducers (Fig. 2B and C, Table 2), but poor for elastic arteries for all transducers studied (Fig. 2E and F, Table 2). Elastic arterial AT and IMAT were systematically overestimated by ultrasound due to difficulties in defining the interface of the adventitia to the surrounding tissue.

Excellent correlations were observed between the entire range of very-high frequency transducer derived measurements of MT (Pearson correlation coefficient range 0.94–0.99), IMT (range 0.95–0.99), AT (range 0.75–0.93) and IMAT (range 0.98–1.0), respectively. In rabbits, MT was slightly underestimated whereas IMT was overestimated with the 25-MHz transducer. Furthermore, the 25 MHz transducer was unable to distinguish the AT in small rabbit muscular artery vessels (IMAT median 0.21 mm) due to fusion of the external elastic layer and the external border of the vessel wall in the image. In larger muscular pig arteries AT and IMAT were overestimated with 12 MHz transducer when compared to the 25–55 MHz transducers. An association between probe-frequency and IT was observed with an increase in IT with decreasing transducer frequency (Tables 1 and 2).

3.5. Precision of thickness measurements in vivo

The borders of the different arterial wall layers in a large number of different muscular arteries and the carotid artery were identified from images obtained from human subjects (Fig. 3). The ultrasound appearance of muscular arteries was similar in vivo compared to in vitro and displayed the triple line pattern, as mentioned above and shown in Fig. 1. In contrast, the carotid artery displayed a dou-

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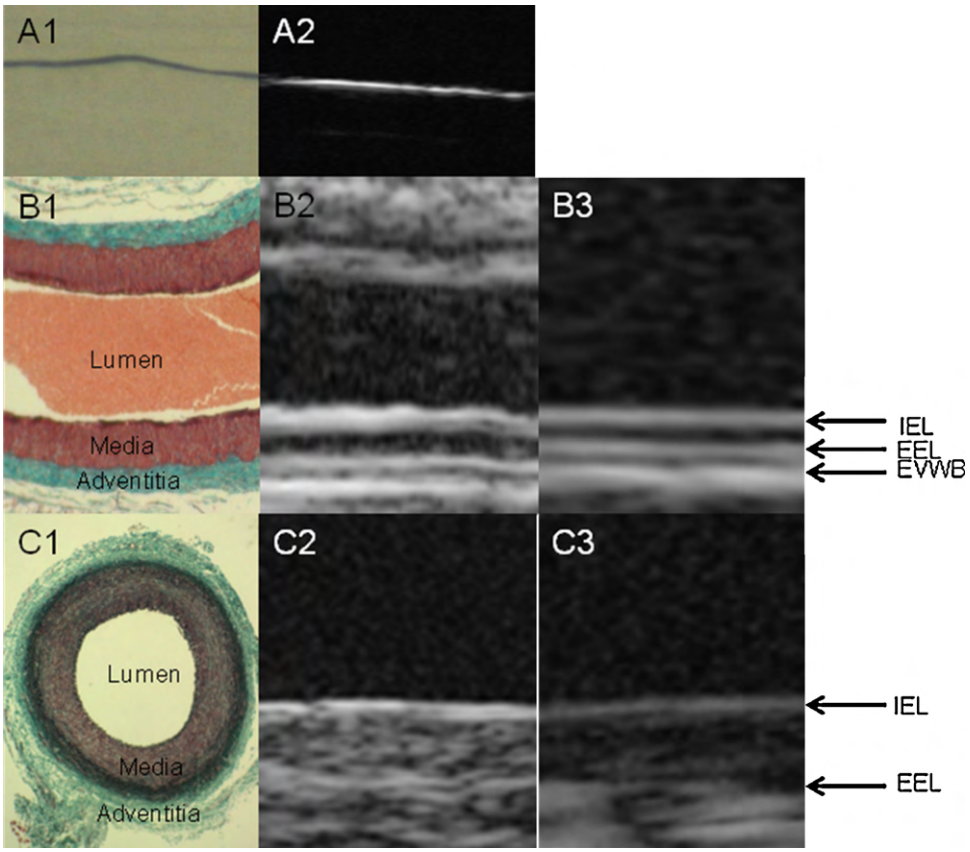

Fig. 1. The histology of an elastic membrane (A1; thickness 0.012 mm), muscular rabbit artery (B1; femoral artery) and elastic pig artery (C1; carotid artery) compared to VHRU images of the corresponding specimens (A2; B2; C2. 55 MHz probe). Magnification of in vivo VHRU images of muscular (B3; radial artery, 55 MHz) and elastic (C3; carotid artery, 35 MHz) arteries of a 5-year-old boy are shown for comparison. Human and animal muscular arteries displays a triple line pattern consistent with the internal elastic lamina (IEL), the external elastic lamina (EEL), and the external vessel wall border (EVWB). The elastic femoral artery displays a double line pattern consistent with the IEL and EEL only together with elastic fibers in the media. Histological staining for muscle in red, internal and external elastic lamina and elastic fibers of the media in black, and collagen in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ble line pattern similar to the in vitro imaging using the lower frequency transducers (Figs. 1 and 3).

The intra- and interobserver variability and test-retest variability in measuring IMT, AT, IMAT and the diastolic lumen dimension with 25, 35 and 55 MHz transducers are shown in Supplement 1. The most superficial peripheral arteries (radial, dorsal pedal, ulnar and brachial) were easily visualized in all subjects using 35 and 55 MHz transducers, but we were not able to visualize the other arteries in all the adults (Supplement 1). No difference was observed for right and left-sided arteries (results not shown). The 95% limits of agreement of wall layer measurements were in the range of less than ±0.05 mm for 35 and 55 MHz (muscular arteries), and in the range of less than ±0.15 mm for 25 MHz (carotid and femoral arteries) as shown in Supplement 1. No bias between the observers was found, except for carotid IMT which was due to discrepancies in delineating the media to adventitia border.

The appearance and thickness of the IT obtained with the 25, 35 and 55 MHz transducers in vivo were identical to the IT appearance in vitro and to artificial elastic membranes as shown in Figs. 1 and 3. This is consistent with a significant overestimation of the ultrasound assessment of the IT in vivo. No differences in IT were observed between the different peripheral muscular arteries. An association between transducer frequency and IT was observed in vivo with an increase in IT with decreasing transducer frequency. The mean IT was 0.064 mm (range 0.06–0.08 mm, $N = 34$) with the 35 MHz and 0.050 mm (range 0.03–0.06 mm, $N = 66$) with the 55 MHz transducer. The mean IT of the carotid artery was 0.064 mm (range 0.06–0.08 mm, $N = 33$) with the 35 MHz and 0.111 mm (range 0.09–0.13 mm, $N = 30$) with the 25 MHz transducer.

3.6. Comparison of VHRU and HRU in vivo

No differences between left and right arteries were observed (results not shown). There was good agreement between conventional HRU with the 12 MHz probe and VHRU with the 25 MHz probe in measuring carotid IMT in healthy adolescents (Supplement 2) with a mean difference of −0.013 mm (95% LOA −0.144 to 0.118 mm, $N = 106$). With the 35 MHz probe, however, we were only able to visualize the near-wall of the carotid artery due to limitations in penetration. The near-wall IMT appeared thicker on 35 MHz-recordings when compared to far-wall IMT measurements obtained with the 25 MHz transducer with a mean difference of 0.019 mm (95% LOA −0.087–0.125 mm, $N = 95$) and 5% proportion of the mean ($p < 0.001$).

4. Discussion

To the best of our knowledge, this is the first study to compare noninvasive VHRU to conventional HRU in the assessment of arterial wall layers in vivo and in vitro. We demonstrate that VHRU is an accurate and precise tool in the assessment of IMT, AT and IMAT in a wide range of normal elastic and muscular arteries. However, contrary to previous reports [15,20], IT appeared grossly overestimated.

The feasibility, precision and clinical utility of assessing carotid IMT with conventional ultrasound imaging have been well established and the method has gained wide acceptance as a clinical research tool in the adult population in particular. The potential

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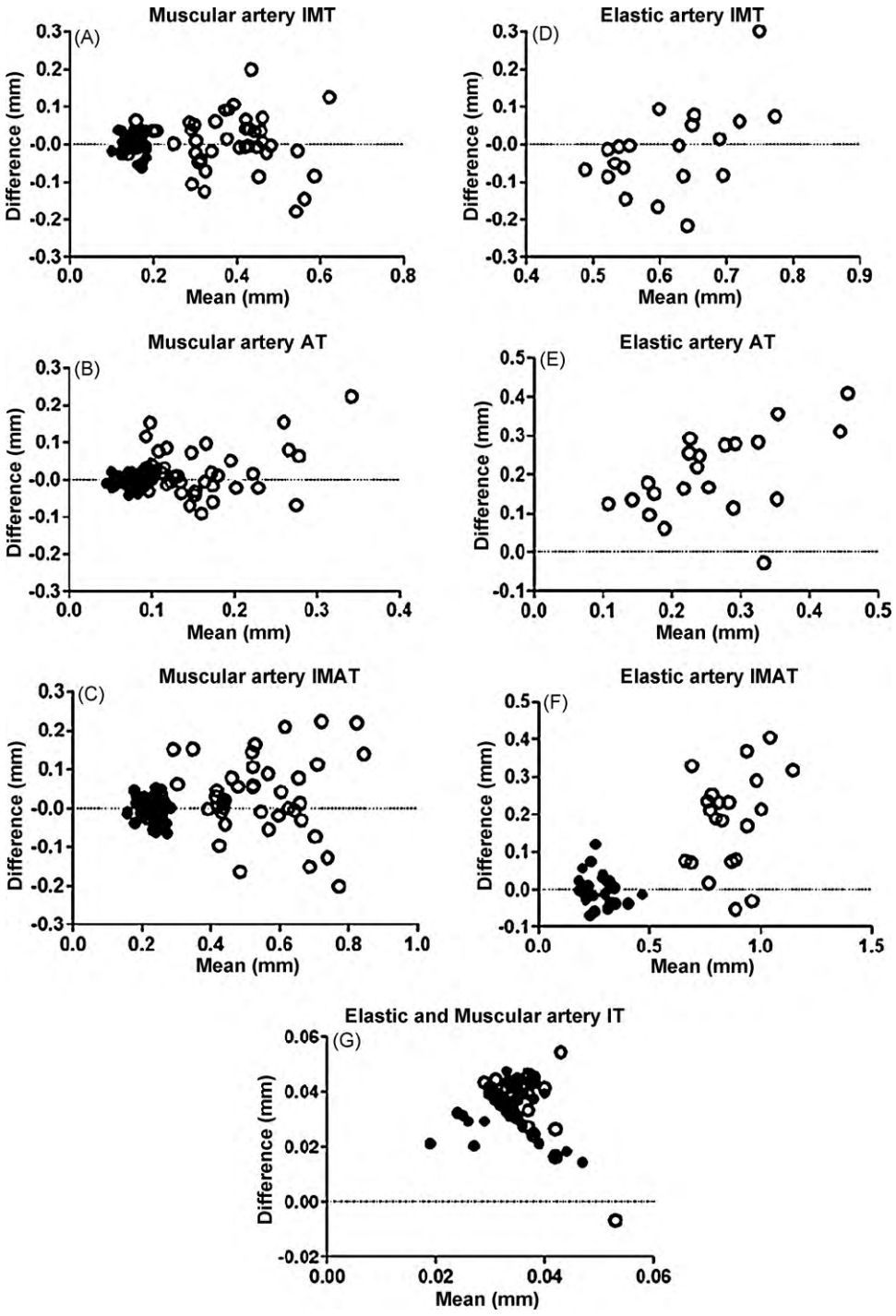

Fig. 2. Bland–Altman plots between VHRU (55 MHz) and histology in rabbits (closed circles) and pigs (open circles). IMT, intima-media thickness; AT, adventitia thickness; IMAT, intima-media-adventitia thickness; IT, intima thickness.

advantage of VHRU is that it may also be useful in examining elements of the vessel wall other then IMT and in imaging the more superficial muscular arteries in almost microscopic detail. A knowledge of, and direct comparison with, the histology of different types of vessel is crucial to the understanding of this 'new' information. Our results significantly expand the concept from the "double line pattern" of the ultrasound image of the carotid artery originally described by Pignoli et al. [5] using HRU to the triple line pattern of the muscular arteries observed with VHRU (Figs. 1 and 3). Our interpretation of the triple line pattern of the far wall ultrasound image is thus based on the following sequence, which is similar to that previously reported for intravascular ultrasound [21,22]: ultrasound reflection is generated when there is an abrupt change in acoustic impedance. The first reflection is generated by the blood-to-intima interface and is likely related to the internal elastic lamina in non-diseased arterial vessels. The media of muscular arteries is relatively echolucent but appears echodense in elastic arteries due to multiple elastin fibers. Our findings show that these fibers are best visualized with the highest of the very-high frequency transducers. The second major reflection appears to be generated by the external elastic lamina at the media to adventitia interface. The third major reflection is likely generated by the interface of adventi-

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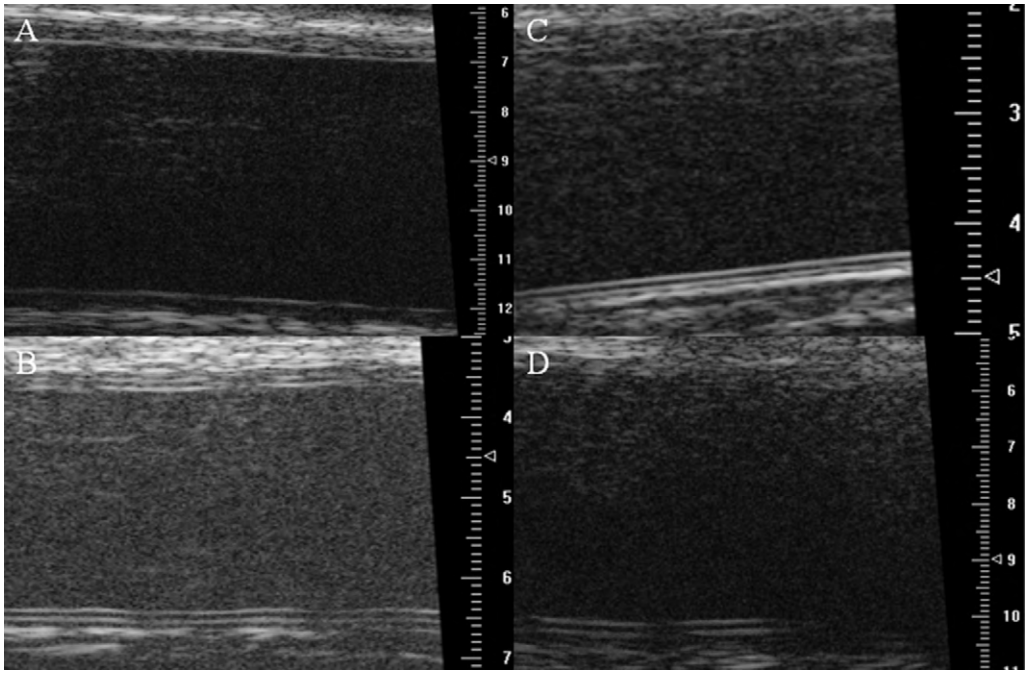

Fig. 3. In vivo transcutaneous VHRU of the carotid artery (A, 35 MHz), brachial artery (B, 55 MHz), radial artery (C, 55 MHz), and femoral artery (D, 35 MHz) in a 5-year-old boy. The scale is in millimeters. The carotid artery displays the double line pattern while a triple line pattern is seen in all muscular arteries.

tia to the surrounding tissue. The ultrasound assessment of carotid IMT has been previously shown to be accurate with HRU [5,8] and is confirmed by our findings. Others have suggested that the area between the first and the second reflection zone which is observed in carotid arteries would be more closely related to the total wall thickness (including the adventitia) of the carotid artery [6] or show a consistent overestimation of IMT by ultrasound [7]. These earlier results on adult carotid specimens imaged with conventional ultrasound are similar to our results in small rabbit elastic arteries using VHRU. It is then clear that some uncertainty in assessing the different layers of the carotid artery of neonates still remain.

This is the first validated method for the noninvasive ultrasound measurement of IMT and AT of peripheral muscular arteries. So far studies addressing IMT have been for the carotid artery mainly in adults and older children [23]. Our results show that in these age groups VHRU ultrasound provides no obvious additional benefits when compared to HRU. Whether there is an advantage of VHRU in the IMT measurement in the smallest children is unknown. However, in this study we examined the accuracy of HRU and VHRU in relatively small vessels with a wall thickness of about 0.2 mm. This is far lower than previously reported validations [5–9], and below the limit of resolution of conventional ultrasound imaging. Indeed, we were unable to use HRU in the smallest of vessels including the more peripheral muscular arteries (Supplement 1), because of poor resolution, precluding analysis. Furthermore, VHRU provides not only higher resolution but also the possibility of assessing more superficial structures in the near field. VHRU provides, therefore, the opportunity to accurately assess the morphology of the elastic and muscular arterial wall in humans of all sizes, including newborns. The selection of the optimal transducer frequency is ultimately a balance between resolution and penetration. Our findings show that IMT and AT of muscular arteries may be accurately and precisely assessed with 35–55 MHz transducers, while the resolution of the 25 MHz limits the assessment of AT in the smallest arteries, and the 12 MHz transducer is only able to distinguish the IMT in relatively large vessels. Although no obvious differences in the accuracy or precision of the thickness measurements were observed using transducers between 35 and 55 MHz, measurements obtained with the highest frequency transducers seem to be more precise, accurate and reproducible. We, therefore, recommend the use of the highest frequency transducer that allows the imaging of the arterial wall of interest without compression from overlying structures.

A major limitation of HRU and VHRU is the inability to image the intima. The IT of the non-diseased medium-sized artery typically measures less than 50 μm [24,25] which is beyond the limit of resolution of the frequencies applied, even with VHRU. In our study the ultrasound assessment of IT was not accurate. Our findings regarding the overestimation of the intima and adventitia thickness are not surprising, and consistent with earlier studies of adult carotid and femoral arteries from human cadavers using conventional ultrasound and intravascular very-high frequency imaging [9,26]. The overestimation of the adventitia in the elastic artery might be related to the loss of the echo from the adventitia to periadventitia interface due to echoes produced by the lower parts of the adventitia. Furthermore, we show a significant interaction between IT and transducer frequency, leading to increasing overestimation of IT with decreasing transducers frequencies. In fact, in our study the IT always measured about three pixels irrespective of the VHRU transducer frequency, which is expected for structures beyond the limit of resolution. Our IT results are similar in magnitude to previous HRU [26] and VHRU [15] studies. Artificial elastin membranes were used in the present study to explore the acoustic properties of elastin compared with the use of silicone phantoms reported by Osika et al. [15]. Furthermore, we assessed the accuracy of ultrasound IT against non-diseased vessel histology compared with Osika et al. who used histology of diseased vessels with an intimal thickness (i.e. 150–400 μm) which, as shown in their report, is beyond the physiological range of peripheral artery IT. Our results are, thus, in line with these previous reports, and the difference in the interpretation of the image explained by these differences in the validation process. These results suggest that the thickness of the first and second echo reflections is unrelated to the true histological thickness of the elastic lamina and, therefore, we recommend to use these reflections as leading edges in the measurement of combined layer thickness, as previously described [5,16].

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Table 1
Accuracy of in vitro ultrasound measurements of small rabbit artery specimens.

Layers	Histology	55 MHz			40 MHz			35 MHz			25 MHz		
		Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%
Elastic artery (n = 25)													
IT	0.020 ± 0.009	0.030	(0.014,0.047)	152*	0.040	(0.022,0.058)	202*,†	0.042	(0.023,0.062)	212*,†	0.082	(0.054,0.111)	411*,†,‡
MT	0.185 ± 0.077	nd		–	nd		–	nd		–	nd		–
IMT	0.205 ± 0.082	nd		–	nd		–	nd		–	nd		–
AT	0.073 ± 0.016	nd		–	nd		–	nd		–	nd		–
IMAT	0.283 ± 0.077	−0.001	(−0.081,0.078)	0	0.006	(−0.077,0.089)	2	0.004	(−0.077,0.086)	2	0.031	(−0.056,0.118)	11*
Muscular artery (n = 53)[a]													
IT	0.013 ± 0.003	0.037	(0.029,0.044)	288*	0.048	(0.038,0.058)	373*,†	0.049	(0.039,0.058)	381*,†	0.090	(0.063,0.117)	706*,†,‡
MT	0.137 ± 0.023	−0.035	(−0.082,0.013)	−25*	−0.038	(−0.086,0.010)	−28*	−0.039	(−0.091,0.014)	−28*	−0.065	(−0.110,−0.019)	−47*,†,‡
IMT	0.150 ± 0.153	0.002	(−0.043,0.047)	1	0.010	(−0.037,0.057)	7	0.010	(−0.041,0.061)	7	0.020	(−0.030,0.071)	14*,†,‡
AT	0.076 ± 0.016	−0.004	(−0.034,0.027)	−5	−0.008	(−0.043,0.028)	−10	−0.004	(−0.038,0.031)	−5	0.008	(−0.054,0.070)	10
IMAT	0.225 ± 0.036	−0.002	(−0.057,0.053)	−1	0.002	(−0.052,0.056)	1	0.006	(−0.056,0.068)	3	0.020	(−0.087,0.127)	9*

Units are millimeters (mm) and values are reported as mean ± SD for histology, mean difference comparing ultrasound with histology and 95% limits of agreement [mean Δ (95% LOA)], and proportion of the mean (%).
Elastic artery includes aorta and carotid artery. Muscular artery includes proximal brachial, renal, and femoral arteries.
Layers include IT, intima thickness; MT, media thickness; IMT, intima-media thickness; AT, adventitia thickness; IMAT, intima-media-adventitia thickness. nd = not detectable.
* $p < 0.0001$ compared with histology.
† $p < 0.0001$ compared with 55 MHz.
‡ $p < 0.0001$ compared with 35/40 MHz.
[a] $n = 27$ for MT, IMT, and AT for 25 MHz due to limited resolution in smallest rabbit vessels.

Table 2
Accuracy of in vitro ultrasound measurements of large pig artery specimens.

Layers	Histology	55 MHz			40 MHz			35 MHz			25 MHz			12 MHz		
		Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%	Mean Δ	(95% LOA)	%
Elastic artery (n = 21)																
IT	0.016 ± 0.007	0.039	(0.023,0.054)	236*	0.051	(0.033,0.069)	312*,†	0.053	(0.033,0.073)	323*,†	0.101	(0.070,0.131)	616*,†,‡	0.140	(0.103,0.176)	854*,†,‡,§
MT	0.606 ± 0.073	−0.055	(−0.280,0.170)	−9	−0.019	(−0.299,0.262)	−3	−0.003	(−0.300,0.293)	−1	−0.040	(−0.341,0.261)	−7	−0.055	(−0.356,0.246)	−9
IMT	0.623 ± 0.073	−0.016	(−0.239,0.207)	−3	0.033	(−0.248,0.313)	5	0.050	(−0.251,0.352)	8	0.061	(−0.240,0.363)	10	0.085	(−0.236,0.401)	14
AT	0.160 ± 0.082	0.193	(0.007,0.392)	120*	0.200	(0.004,0.396)	125*	0.196	(−0.005,0.397)	123*	0.232	(0.016,0.447)	145*	0.197	(−0.033,0.427)	123*
IMAT	0.759 ± 0.123	0.183	(−0.066,0.432)	24*	0.207	(−0.042,0.457)	27*	0.200	(−0.099,0.498)	26*	0.247	(−0.079,0.573)	33*	0.245	(0.030,0.461)	32*
Muscular artery (n = 41)																
IT	0.016 ± 0.007	0.036	(0.020,0.052)	223*	0.049	(0.028,0.070)	307*,†	0.050	(0.027,0.073)	312*,†	0.094	(0.069,0.119)	587*,†,‡	0.141	(0.092,0.191)	884*,†,‡,§
MT	0.365 ± 0.120	−0.031	(−0.172,0.111)	−8	−0.038	(−0.184,0.107)	−10‖	−0.046	(−0.206,0.113)	−13*	−0.063	(−0.205,0.079)	−17*	−0.105	(−0.286,0.076)	−29*
IMT	0.481 ± 0.122	0.005	(−0.141,0.151)	1	0.011	(−0.142,0.164)	3	0.001	(−0.168,0.169)	0	0.031	(−0.121,0.183)	8	0.036	(−0.144,0.216)	9
AT	0.142 ± 0.066	0.024	(−0.108,0.157)	17	0.026	(−0.110,0.162)	18	0.035	(−0.098,0.168)	25	0.053	(−0.103,0.208)	37*	0.094	(−0.052,0.239)	66*,†,‡,§
IMAT	0.523 ± 0.161	0.030	(−0.169,0.228)	6	0.037	(−0.172,0.246)	7	0.039	(−0.190,0.268)	7	0.076	(−0.139,0.291)	15*	0.130	(−0.109,0.369)	25*,†,‡,§

Units are millimeters (mm) and values are reported as mean ± SD for histology, mean difference comparing ultrasound with histology and 95% limits of agreement [mean Δ (95% LOA)], and proportion of the mean (%).
Elastic artery includes carotid artery only. Muscular artery includes proximal brachial, renal, and femoral arteries.
Layers include IT, intima thickness; MT, media thickness; IMT, intima-media thickness; AT, adventitia thickness; IMAT, intima-media-adventitia thickness.
* $p < 0.0001$ compared with histology.
† $p < 0.0001$ compared with 55 MHz.
‡ $p < 0.0001$ compared with 35/40 MHz.
§ $p < 0.0001$ compared with 25 MHz.
‖ $p < 0.01$ compared with histology.

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4.1. Study limitations

This study was designed to assess the accuracy and precision of noninvasive VHRU in examining normal healthy subjects only. The feasibility of VHRU will need to be further evaluated in larger populations and different age groups as well as in subjects with different cardiovascular conditions. Ultimately its clinical utility and usefulness in the assessment of peripheral muscular arteries, in particular in response to interventions will also need to be tested. In addition, we assessed the accuracy using specimens obtained from animals only, and did not check for changes between in situ and in vitro imaging of the vessels as excellent agreement regarding this comparison has been reported before [6]. Furthermore, the minimal shrinkage of the formalin fixative on the layer thicknesses was similar to previously reported [9] and so our data are likely to be relevant to in vivo accuracy.

In conclusion, we present a noninvasive very-high-resolution ultrasound imaging method to assess the combined intima-media, adventitia and total wall thickness of superficial elastic and muscular arteries that is beyond the resolution of conventional ultrasound imaging techniques. The technique will therefore likely provide new insights into the pathogenesis of vascular disease in adults and children.

Conflicts of interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.06.043.

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