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Title page:

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**TRANSMISSION SCANNING ACOUSTIC IMAGING OF HUMAN CORTICAL
BONE AND RELATION WITH THE MICROSTRUCTURE**

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ABSTRACT

Objective: To investigate and compare the spatial distribution of velocity with that of the microstructural properties (dimension of the haversian canal, percentage of porosity) on cross section of cortical bone.

Design: Experimental investigations permitted to quantify variation of acoustic properties related with that of the microstructural properties.

Background : Transmission ultrasonic techniques have been used in vitro and in vivo to assess the elastic and acoustic properties of Human bone, but few investigated the relationship between their variation with that of the microstructure.

Methods: Two scanning techniques (in transmission with a focused transducer at 5MHz and an environmental scanning electronic microscope at 20KV) enabled to obtain the spatial distribution of relative acoustic velocities and the microstructural properties (pore size and porosity).

Results: Increase of the velocities is related with the decrease of pore size and porosity. Around the periphery of the sections, the velocities were found to be significantly lower in the posterior side with a significant increase along the length. Radial variations are correlated to the spatial distribution of the microstructure where the endocortical region is more porous compared to the periosteal region.

Conclusion : Significant alterations of the microstructural properties of the cortical bone reflect small variation of velocity suggesting that the velocities are not so sensitive to microstructural changes.

RELEVANCE

These results are of importance for the clinicians and researchers to get a better understanding (advantages and limitation) of the use of ultrasound technique to assess material and structural properties of cortical bone. Our study suggested that velocity could be an index of porosity.

Then it would be of interest to improve the clinical assessment of bone quality by describing bone both by a mineralization index (BMD) and a microstructural index.

Keywords:

Human Cortical Bone - Ultrasound – Porosity – microstructure

1. INTRODUCTION

Ultrasonic technique is a non-invasive technique, which enables the assessment of acoustic properties (velocity, impedance, attenuation) and elastic properties (Young's modulus, Shear modulus, Poisson's ratio) of a solid material. In Human bone, transmission ultrasonic technique has been used in vitro to measure elastic properties of cortical bone. Typical contact transducers with a frequency of 2.25MHz were used (Ashman et al., 1984; Ho Ba Tho et al., 1991). Thus, bulk velocities were measured in the longitudinal and transversal directions in long bones (femur, tibia). The range of the bulk velocities for all directions provided in the literature varies from 3400 to 4000m/s (Bensamoun et al., 2002).

Another technique called the scanning acoustic microscope (SAM) scans the surface of the bone, which enables to obtain the acoustic impedance. Thus, some authors (Meunier et al., 1988; Weiss et al., 1998) used this technique which operates in reflection with low frequency between 20MHz and 50MHz in order to have a mapping of the acoustic impedance on Human femur cross sections which itself supplies information at the microstructure level.

It is well known that the microstructural properties reflected by the porosity (blood channel or pores of the osteon) and elastic properties of the osteon, interstitial lamellae, influence the elastic properties of the human cortical bone. Therefore, different histological techniques and imaging techniques have been used in order to provide a relationship with the elastic

properties of human cortical bone. A study has concluded that 80% of the variation of the elasticity of the cortical bone is due to the porosity and the volume fraction (Currey et al., 1988). In the same way, another study investigated mechanical test in tension on cortical femoral and tibia samples from animal (Steers with an age of 2-3 years) (Schaffler et al., 1988). Then, the ash weight and the porosity (with the point-counting technique) of the samples have been determined. A stronger relationship has been found between the elasticity and the volume fraction ($R^2=0.86$) compared to the elasticity and the ash weight ($R^2=0.64$).

Other authors (Ascenzi et al., 1967; Ascenzi et al., 1968; Frasca et al., 1976; Martin et al., 1989) add to the previous parameters (i.e. porosity and mineralization) the effect of the lamellar collagen orientation. The total porosity for the osteonal group (0.0680 (SD 0.008)) is lower to the plexiform (0.103 (SD 0.014)) and mixed group (0.0924 (SD 0.014)). The longitudinal structure index (LSI) defined by the ratio between the bright field and the dark field through a polarized microscope shows a higher index respectively for the plexiform (20.6 (SD 5.2)) and mixed structure (22 (SD 5.9)) compared to the osteonal structure (13.3 (SD 4.5)). This last observation is normal due to the lamellar structure of the plexiform bone.

It was concluded that the prediction of the elasticity is better if many histological parameters have been measured; furthermore the LSI parameter is also important (like porosity, mineralization and density) for the determination of the elasticity.

Other authors (Watcher et al., 2001) used imaging technique like micro computed tomography (μ CT) in order to predict the elastic properties of the cortical bone. A correlation ($R^2=0.64$) has been found between the percentage of porosity (range of 2% until 32%), determined with the μ CT and the yield stress, and $R^2=0.45$ between the porosity and the elastic modulus. The porosity alone cannot explain the mechanical properties. However the BMD (bone mineral density), determined also with the μ CT which depends on the porosity and the mineral content, has a strong correlation in this study with the mechanical properties

($R^2=0.72$ with the yield stress and $R^2=0.67$ with the elastic modulus). This study shows the importance of the BMD ($950\text{mg/cm}^3 < \text{BMD} < 1400\text{mg/cm}^3$) for the determination of the mechanical properties. The BMD, which is a non destructive method for the estimation of the elasticity in vitro, allowed to have a predictive value for the microstructural properties (porosity, dimensions of the pores, density of the osteons), which are strongly correlated to the mechanical properties of cortical bone (Watcher et al., 2002).

Thus, the literature review shows that ultrasonic method as well as in transmission (with contact or focused transducers) as in reflection (with the scanning acoustic microscope, SAM) is able to determine the elastic properties. Furthermore, the spatial distribution of impedance with the SAM enabled to observe the inhomogeneities through the cross section.

Histological parameters (like porosity or volume fraction, degree of calcium content, density of osteon) were assessed to predict the Young's modulus.

The objective of this work is to correlate the spatial distribution of acoustic properties of Human cortical bone with its microstructural properties such as pores diameter and porosity.

In order to achieve the objective, spatial distribution of velocities and microstructure properties will be quantified on the same cross sections of human femoral cortical bone. The variation between the acoustic properties and microstructural properties will be compared in order to correlate objectively their spatial distribution.

2. MATERIALS AND METHODS

2.1. Specimens preparation

A cadaveric femur (male 76 years of age) was obtained from the anatomical laboratory of Hospital University of Amiens. Four cross-sections have been cut between 40% and 70% of the total length of the femur. Each slice has been sectioned transversely with a low-speed diamond saw (MICROCUT 2, Hergon, Argenteuil, France) and water has been used to cool the blade. Their identification is related to the level of the total length of the femur from proximal to distal, section 1 (45%), section 2 (50%), section 3 (56%), section 4 (60%).

2.2. Thickness measurements

The measurement of the thickness around the periphery of the section was performed with a digital micrometer (Mitutoyo, 293, Tokyo, Japan). The accuracy of the measurement is 0.01mm and the diameter of the tip is about 3mm. A total of 12 measurements were performed around the periphery of each cross section. The spatial distribution allowed checking the uniform thickness of the surface i.e to verify the parallelism between the faces of the cross section.

2.3. Determination of reference frame for mapping ROI from SEM and acoustic velocities mapping.

All the cross sections have been automatically scanned on X-Y directions with an optical microscope (Leica, DM LB 11888010, Wetzlar, Germany) in transmission at a magnification of x20. Calibration was performed with a Mallassez cell allowing the real size of femoral sections to be obtained. A rectangular frame was then calculated by fitting four cardinal

points of the cross section. This frame was then used as a reference coordinate frame for SEM (scanning electronic microscope) and acoustic velocities mapping.

2.4. Scanning transmission imaging ultrasonic technique

Measurements of velocities will be determined through an ultrasonic method, which operates in transmission (fig.1). The ultrasonic equipment is composed of a generator (the bandwidth is 40MHz) (Sofranel 5052, Atlanta, GE, USA), which delivers electrical pulses. The pulses are transmitted to the piezoelectric transducer, which converts the electric signal into an acoustic wave. The transducer is characterized firstly by its shape and secondly by its parameters. The shape of the transducer is spherically and focused which works in immersion; the frequency of the transducer is 5MHz (Panametrics, V309, Houston, TX, USA) with a focal length of 75mm and a diameter of the spot (Beam width) of 1.24mm. The wavelength at 5MHz is 0.8mm. A part of the acoustic wave is reflected back to the transducer acting as a receiver, which transforms the acoustic wave into an electrical pulse in order to obtain longitudinal acoustic velocities on each femoral section. Secondly, through an oscilloscope (LeCroy 9410, Chestnut Ridge, NY, USA) with a vertical resolution of 8 bits and a sampling frequency of 200MHz it is possible to visualize the amplitudes of the different echoes which were propagated through the samples.

Lastly, the scanning system is controlled by a microcomputer, which uses custom-made software MICROXYZ (Controller Driver MM 2500 Version 2.0 User's Manual, Newport, Fairport, NY, USA) to displace the focused transducer in order to obtain many measurements on the surface of the specimen. The computer in the X, Y and Z directions drives the transducer with respectively an accuracy of 1 μ m, 0.1 μ m and 1 μ m and a step of 0.5mm. The first stage has been to use the MICROXYZ software to adjust the parallelism between the surface of the transducer with the bottom of the container. Then the Z direction is translated in

order to focus the signal on the cross section. An automatic scanning on X-Y direction is performed with a step of 0.5mm. Each section has been laid down in demineralized water ($T=20^{\circ}\text{C}$) into a little container which is mounted on an antivibration table. On each point of acquisition, two acoustic echoes have been recorded: the first one between the water and the material (echo 1), the other one between the material and the water (echo 2) (fig.2). The process to measure the time delay between the two different echoes is the following:

The window of the oscilloscope that contains the two signals is divided into two windows composed each one of one signal. Then, on each part of the windows the maximum and minimum values of each signal is detected. The time delay of the first zero crossing between the two echoes was measured.

The acquisition has been performed through the software MICROXYZ with an averaging of 10 in order to have a good quality of signal. The number of points of acquisition varies from 1460 to 1734. The time delay between the two echoes is measured with a custom made software CACTUS (CarACterization of an ultrasonic transducer) in order to measure the average velocity (m/s) representing all the surface and expressed by the thickness of the cross section and the measured time delay (μs): $v=2e/t$, e being the average thickness and t the time delay. A colour map representing the spatial distribution of velocities has been computed from the software Matlab. The reference coordinate frame previously used with the optical microscope was applied on the cartography of the velocities in order to define region of interest (square of 3 mm length) located at the posterior, lateral and medial regions. This procedure was repeated for each section with appropriate defined reference frame.

The reproducibility of the technique was quantified by repeating all the process of replacement and measurement three times on one sample.

2.5. Determination of microstructural properties derived from Scanning Electronic

Microscope (SEM) images.

After the acoustic measurements each cross section has been ground with sandpaper (grit #1200) and then polished with 0.5 μ m alumina slurry (diameter of 0.3 μ m). An optical microscope in reflection (Leica DMLS, magnification X10) has been used in order to control the polishing. Back – scattered environmental scanning electron micrographs (XL 30 ESEM - FEG) were produced under high vacuum at 20KV with a magnification of x22. This work was performed in the Department of Chemical Physical Analysis at the University of Technology of Compiègne. The advantage of this technique is that none of the specimens was treated on the surface. The samples were scanned with an electron beam in order to have a mapping of secondary electron, which is related to the composition of the microstructure.

Uncompressed Tiff images size obtained were typically (712x484 pixels) with a mean resolution of 5 μ m per pixel. A total of 40 images were obtained for each section.

Then the rectangular frame with the ROI (region of interest) obtained from acoustic velocities mapping was superimposed on the SEM pictures. Thus, the same regions of interest (square size of 3 mm) as those defined on the cartographies of velocities are obtained in the same area (posterior, lateral and medial).

The regions of interest of the SEM images were transferred into a custom made software (Ho Ba Tho., 1993) in order to perform an edge detection of the pores. The geometric data was then transferred into a graphic workstation R5000 (Silicon Graphics, Mountain View, CA, USA), then the cross section areas of the pores were calculated from a pre and post processing computer aided design software Patran V.9 (MSC. Software, München, Germany).

The porosity expressed in percent was defined by the ratio between the total area of the pores divided by the total of the section of interest. The pore size was defined by a diameter D of a mean surface S of a pore calculated as:

$$D = \sqrt{\frac{4S_p}{\pi N_p}} \quad (1)$$

D: mean diameter of the pores in μm

S_p : total surface of the pores

N_p : total number of pores

The accuracy of the methodology was assessed by using calibrated radius circle of 50, 100, 200 and 400 μm and ellipsoids of different length axes (50 μm x 100 μm , 100 μm x 200 μm and 200 μm x 400 μm). The reproducibility was quantified by repeating all the process three times.

2.6. Statistical analysis

Analysis of variance (ANOVA test) and correlation between velocity and porosity has been performed with the software Statgraphics 5.0 (Sigma Plus, Maryland, USA). This would allow to test the significance of the variation of the acoustic and microstructural properties function of the anatomical localization.

3. RESULTS

3.1. Thickness measurements

The result of the average thickness of the four cross sections 1 to 4 were respectively 1.728 (SD 0.054)mm, 1.934 (SD 0.030)mm, 2.142 (SD 0.026)mm, 2.192 (SD 0.025)mm. The measurements performed on section 3 are illustrated Figure 3. The average of the thickness section is about 1.99 (SD 0.02)mm. The maximum variation in the thickness range was found to be 0.086mm for the first section.

3.2. Cartography of velocities

The accuracy of the acoustic velocities is 137m/s. The reproducibility obtained with three measurements is about 140m/s. The time delay error between the two echoes is 10ns.

Mapping of velocities exhibits local variations of velocities if the thickness is assumed to be equal across the sample. Variation of the thickness for each sample has to be considered in order to confirm the significance of local variation. The significant velocity variation will be represented by a change in colour.

In fact, six fringe colours (dark blue, blue sky, green, yellow, orange, red) represent a range of 200m/s. Each colour variation represents a significant variation of velocity, except for section 1 where a change about two fringes was significant (for example blue and yellow) due to the high thickness variation (mean value 0.054mm). A thickness variation of 0.05mm and 0.03mm induces a variation in velocities (ΔV) about 243m/s and 175m/s respectively.

The reconstructed acoustic images of the sections for the four sections obtained at 5MHz are illustrated Figure 4.

Around the periphery, for all the sections, the posterior aspect exhibits significant lower velocities. The range of velocities at the posterior aspect varies from 3400m/s-3800m/s. It represents a velocity variation of 400m/s (variation about 11%). Velocities on the medial aspect seemed to exhibit lower values than those of the lateral aspect and are significant for the section 4. Their range of values is 4000-4200m/s (variation about 5%). Along the length, we can notice only velocities at the posterior aspect which increase significantly from 45% to 60% of the total length of the femur. A significant radial variation is observed, velocities increased from endocortical to periosteal aspect (3800-4200m/s) (variation of 11%).

Globally the velocities vary from 3400-4200m/s according to the anatomical location. The difference of 800m/s represents about 20% of velocity variation.

3.3. Determination of microstructural properties derived from scanning electronic microscope images.

The mapping of the 40 images obtained with SEM for each section is illustrated Figure 4. The region of interest was located for the measurements of the microstructural properties such as pores diameter and porosity.

The accuracy of the technique of measurement of the diameter of the pores is about 10 μ m. The reproducibility of the measurement of the surfaces and the porosity are about 4% (0.2 – 10%) and 0.011 respectively.

The values of the mean pores diameter and the porosity of the region of interest are summarized in Table 1 and Table 2 respectively for each section.

The number of pores of all regions of interest varies from 41 to 132, average number of 70 (SD 23) pores.

The mean values of pore size of the posterior aspect are significantly higher than the lateral and medial aspects (higher than factor 2) ($P<0.01$). The range of pore size for the posterior aspect is (112 (SD 51) μm –253 (SD 243) μm) and the other aspects (47 (SD 17) μm -83 (SD 41) μm).

Furthermore, the posterior aspect exhibits significant difference of the pores diameter for all the sections ($P<0.01$). Similar results are found for the values of the porosity, the range of values for the posterior aspect is (9.7%-47%) and the other aspects (3.1%-5%).

Globally the pore size and the porosity may vary according to the anatomical location about 47 μm to 253 μm and 3 to 47% respectively.

3.4 Comparison between acoustic and microstructural properties

The values of acoustic and microstructural properties measured on the same region of interest (lateral, medial and posterior) of the four sections are illustrated Figures 5 - 6.

The respective range velocities, porosity and pore size varied from 3500 to 4200 m/s, 3.1% to 47% and 47 μm to 253 μm . The variation of velocities is less important (factor 1.2) than the porosity (factor 14) and pore size (factor 5).

The relationship between velocity (m/s) (V) and porosity (%) (P), and the determination coefficient is expressed by the following equation :

$$V = 4185 e^{-0.0042P} \quad R^2 = 0.90$$

4. DISCUSSION

4.1 Range of velocities values obtained and compared to the literature

In a previous study (Bensamoun et al., 2002) it has been demonstrated that the velocities obtained with the focused transducer at 5MHz are close to the absolute velocities obtained with flat transducer. These results are of importance for the comparison with literature values obtained with other ultrasonic techniques. In the present study, the range of velocities (3400m/s – 4200m/s) obtained on the same cross section, presents a larger range (about 800m/s) compared to the literature (range about 600m/s) (Ashman et al., 1984; Ho Ba Tho et al., 1991; Zimmerman et al., 1990). Also, Hasegawa's study with the reflection technique in vitro shows lower velocities (3318m/s – 3663m/s) with a lower range compared to this study due to probably the type of bone (iliac crest biopsy).

4.2 Spatial distribution of velocities according to the anatomical location

Heterogeneities of velocities around the periphery were also found by previous authors (Meunier et al., 1988; Weiss et al., 1998).

In the present study, the significant variation of velocities on the posterior site found along the length confirmed the observation with spatial distribution of acoustic impedance measured with the scanning acoustic microscope (Meunier et al., 1988; Weiss et al., 1998). The significant radial variation of velocities is due to a higher porosity in the endocortical areas, which represents the interface (porous area) between the bone marrow and the cortical bone. Overall, these variations are related to the vascularization environment.

In addition the endocortical region becomes more spongious at the distal portion and especially from the two last sections, near the metaphyseal part (section #4, Figure 4). Thus the presence of these macroscopic pores should reflect a decrease of velocities. In fact we

have increased velocities which are artefacts represented by dark red spots (4600m/s). A previous study has shown that the size of the pore influences the wave propagation and artefacts appear depending on the ratio between the wavelength and the pore size (Bensamoun et al., 2002).

In order to be in the assumption of a continuous media the wavelength should be higher than the longest dimension of the pore. Besides, the presence of macroscopic pores reflects an increase of velocities instead of a decrease, especially around the endocortical region (see the section #4) where a more porous area is located. The attenuation of the second echo implies a random detection of the second echo that is close to the first one. Subsequently, the time delay is decreased and the velocities increase.

4.3 Spatial distribution of microstructural properties

In the present study, the range of the porosity (3-47%) is found to be higher than usual range found in the literature. This may be due to the site location since our purpose was to study the relation between the microstructure with the acoustic measurements according to the anatomical location. Mean value found in the literature is about 10%; the range varies from 2 to 32% (Martin et al., 1989; Watcher et al., 2002). According to our knowledge, no data on mean pore size was available in the literature for comparison.

The significant variations of the pore size and porosity are correlated to the velocities variation. This demonstrated a significant relationship between the changes of acoustic properties with that of the microstructure. But it must be noted that it is not in the same order of variation.

The maximum variation of the velocity is about 800m/s, which represents a variation of 20% of velocity. The variation of pore size is about a factor 5 and porosity about 15 (factor 2 for the bone volume fraction).

So, the variation of velocity is much smaller than the parameters of the microstructures. For example, one can note that there is obviously a strong correlation between the increase of velocity (3400-4000m/s) (Figure 4) in the posterior side with a strong decrease in porosity and pore size. The variation of velocities is about 11%, meanwhile the porosity decreases about a factor 4 (factor 1.7 for the bone volume fraction) and the pore size about a factor around 2.

One should note that the measurements have been performed in the axial direction of the femoral bone and on one specimen. Besides, the range of values is within the atlas of mechanical properties of cortical bone performed on 8 subjects (Ho Ba Tho et al. 1991).

The range of velocities found in our study is in agreement with data from literature. within the range found by several authors. Besides few explanations are provided on the variation of velocities (Hasegawa et al 1995).

For example, Weiss et al. 2000, measure the propagated waves in vivo along the radius and tibia related to the age (20 – 89 years old). Subjects between 20 and 60 years old have velocities between 4022m/s and 4146m/s for the radius and 3809m/s and 3938m/s for the tibia. After 60 years old, a decrease of velocity (between 142m/s and 179m/s) appears as well for the radius (3880m/s – 3967m/s) as for the tibia (3641m/s and 3767m/s).

Our relationship between velocity and porosity would suggest that this decrease of velocities represents an increase of about 10% of porosity. For example, if the initial porosity was about 5%, then after 60 years old it would be about 15%.

In vivo acoustic measurements were performed on ulnar head of premenopausal normal, osteoporosis and postmenopausal women with an ultrasonic reflection technique (Antich et al., 1993). No significant difference of mean velocity was found for premenopausal normal, osteoporosis and postmenopausal women (4123 (SD 174)m/s, 4089 (SD 178)m/s, 4045 (SD

199)m/s). Our relationship between velocity and porosity would suggest that velocity of osteoporotic women (porosity about 40-50%) should be about 3500m/s.

In a technical point of view, this alternative ultrasonic acoustic scanning method enables to perform a spatial distribution of velocities. The results obtained with this experimental design are correlated with the microstructure. Our technique has the advantage to provide direct distribution velocities which are representative of mechanical properties of bone (elasticity combined with density) compared to other studies where the mapping of acoustic impedance doesn't allow a direct measurement of elastic properties (Zimmerman et al. 1990).

5. CONCLUSION

Spatial distribution of acoustic properties of Human cortical bone and that of microstructural properties (pores diameter and porosity) were compared on the same cross sections of human femoral cortical bone. The decrease of acoustic properties is related to the increase of the microstructural properties. Besides, high changes of the microstructural properties reflect small variation of velocities showing that the velocities are not so sensitive to microstructural changes. This suggests high accuracy measurement of the ultrasonic technique. These results are of importance for the understanding of the use of the ultrasound technique for assessing elastic or acoustic properties of cortical bone in vitro and in vivo.

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Tab.1 Range of pores size (mean (SD)) function of the anatomical location.
(S1: section #1; S2: section #2; S3: section #3; S4: section 4)

	Mean diameter Pore size (µm) medial	Mean diameter Pore size (µm) posterior	Mean diameter Pore size (µm) lateral
S1	62 (SD 41)	253 (SD 243)	47 (SD 17)
S2	60 (SD 25)	191 (SD 168)	59 (SD 30)
S3	64 (SD 38)	140 (SD 51)	54 (SD 19)
S4	83 (SD 26)	122 (SD 81)	83 (SD 41)

Tab 2. Values of porosity according to the anatomical location.
(S1: section #1; S2: section #2; S3: section #3; S4: section 4)

Sections	Medial	Posterior	Lateral
S1	3.5%	47%	3.1%
S2	3.4%	40%	4.6%
S3	5%	20%	3.7%
S4	5%	15%	4.6%

Fig 1. Ultrasonic transmission technique with focused immersion transducer

Fig 2. Representation of the echos through the section

Fig 3. Superimposition of cartography of thickness and velocities for the section 3

Fig 4. SEM images and cartographies of velocities for the section #1, section #2, section #3 and section #4.

Fig 5. Regression between velocity (m/s) and porosity (%) ($N = 12$, $R^2 = 0.90$, $P < 0.001$)

Fig 6. Pooled data of porosity (%) and diameter of pores $D(\mu\text{m})$

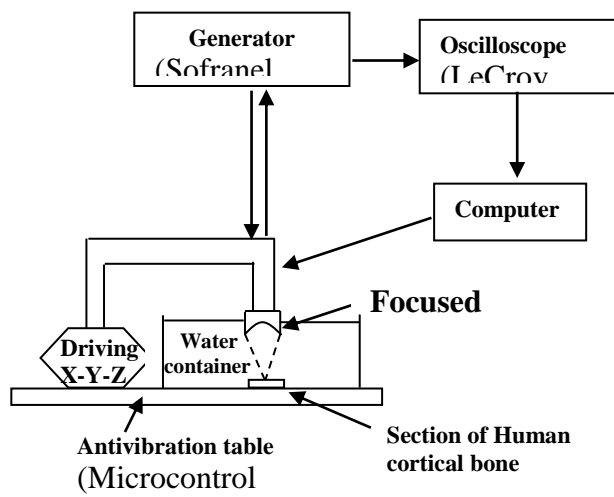
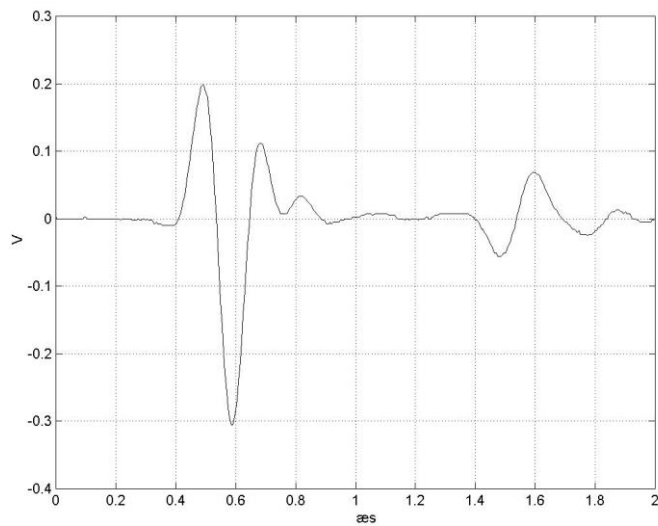
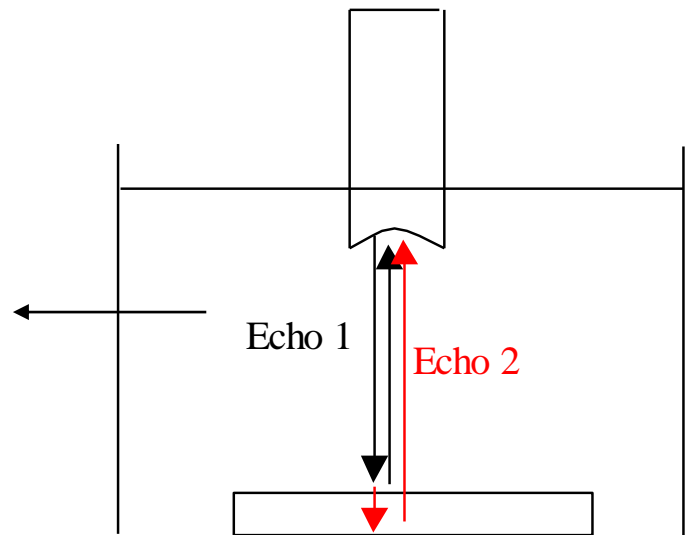
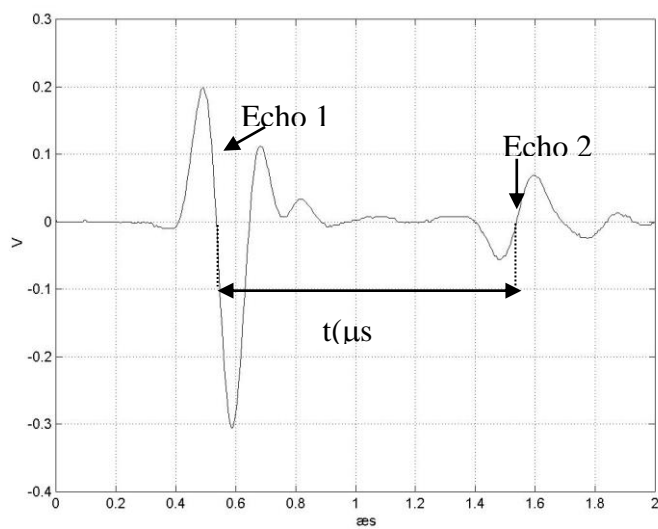


Fig.1 Ultrasonic transmission technique with focused immersion transducer



chos through the section

ie cross section

ss section

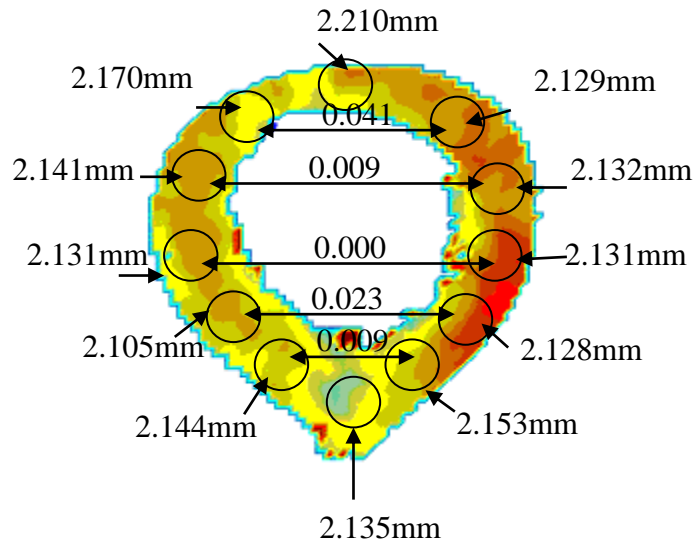


Fig.3 Superimposition of cartography of thickness and velocities for the section 3

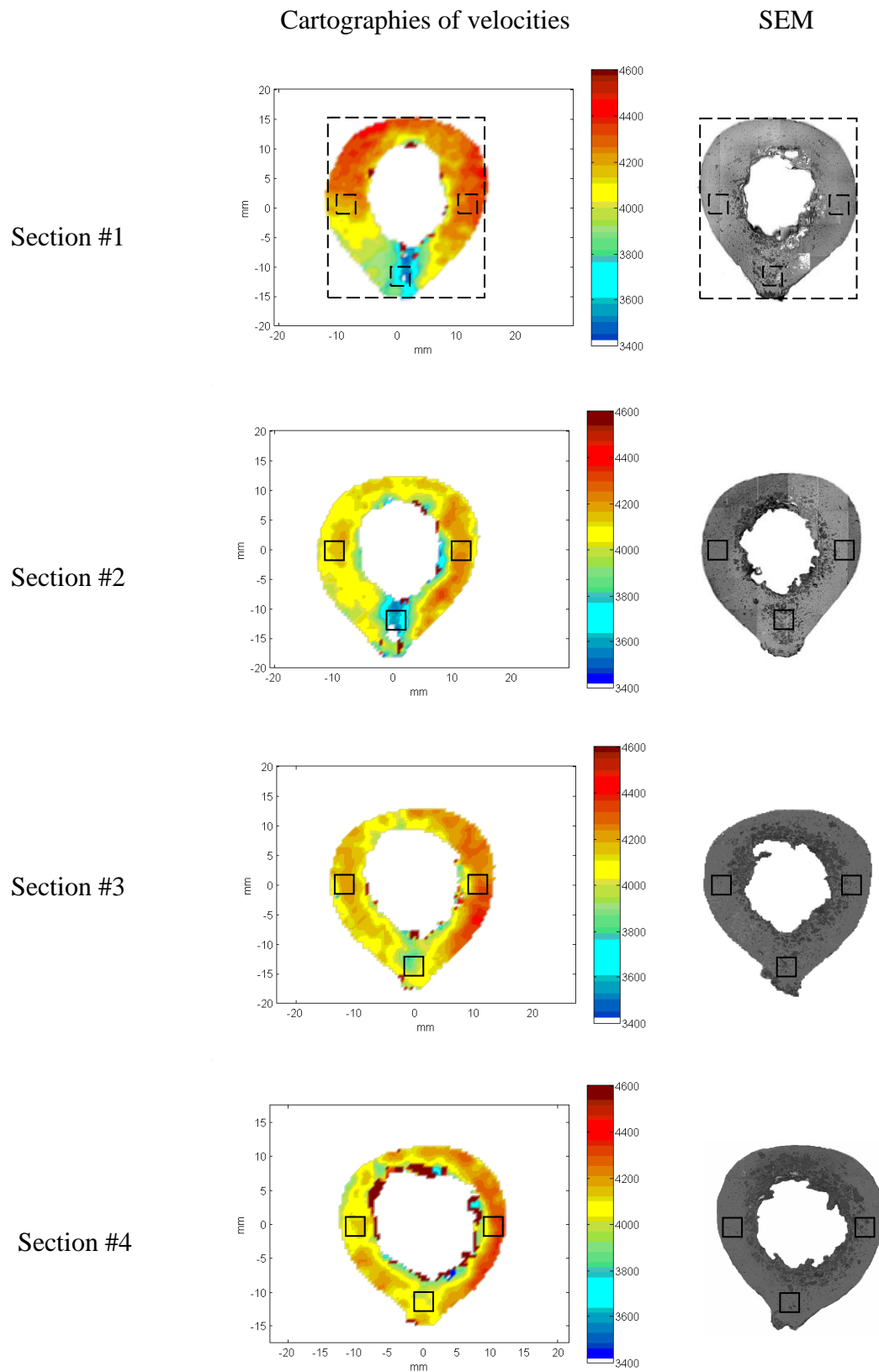


Fig.4 SEM images and cartographies of velocities for the section #1, section #2, section #3 and section #4.

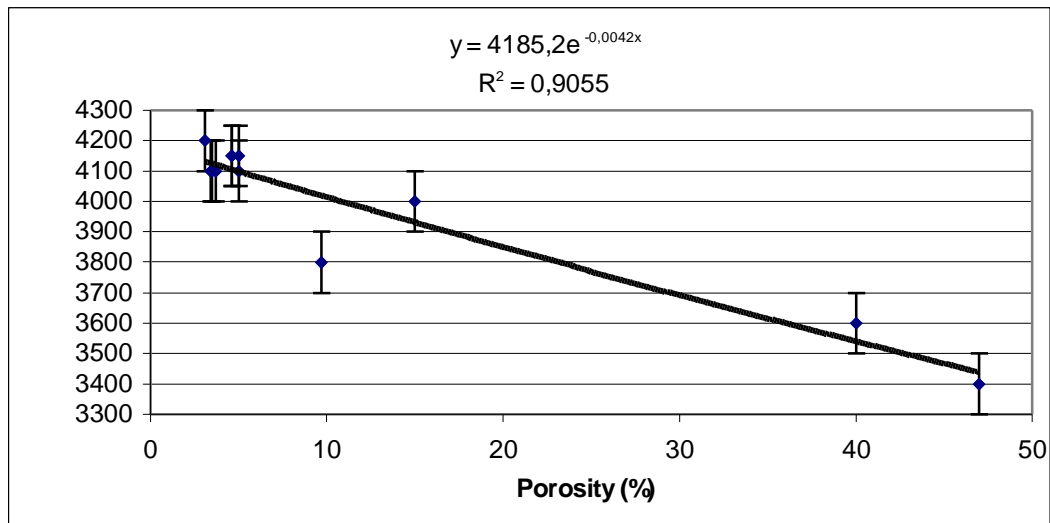


Fig 5. Regression between velocity (m/s) and porosity (%) ($N = 12$, $R^2 = 0.90$, $P < 0.001$)

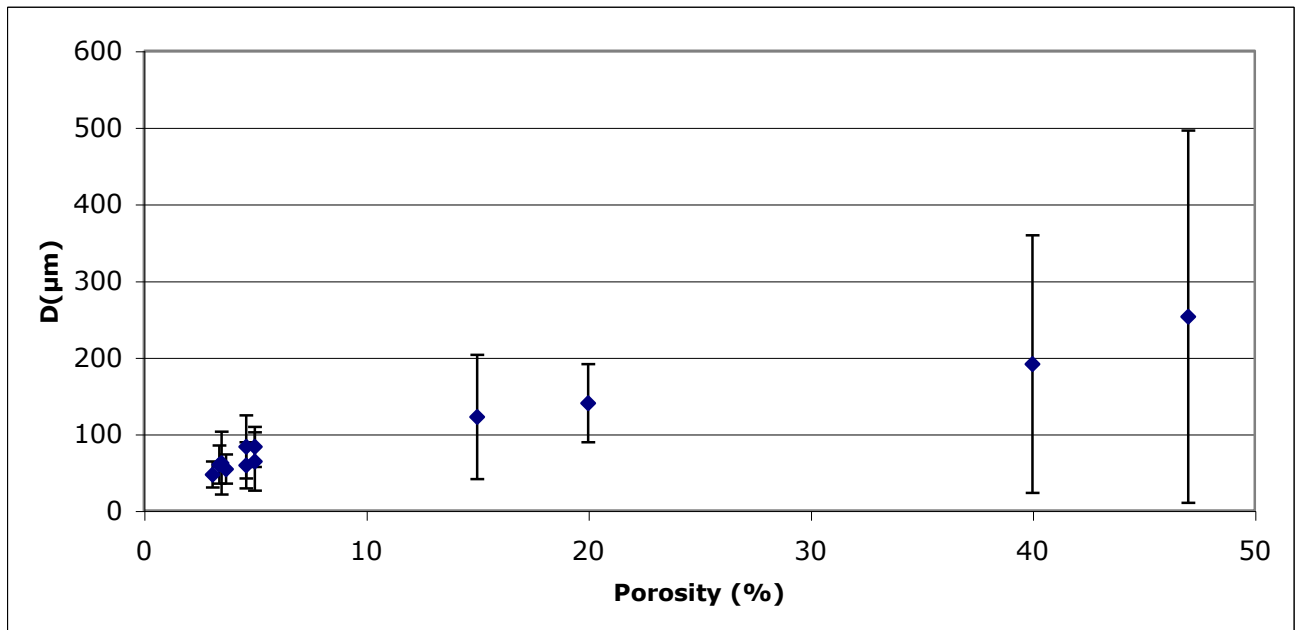


Fig 6. Pooled data of porosity (%) and diameter of pores $D(\mu\text{m})$