Validation of ultrasonography to assess the degree of hereditary cutaneous hyaluronosis in shar pei dogs

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Abstract

Objective: To demonstrate that high-frequency diagnostic ultrasonography could be a useful instrument to assess the degree of hereditary cutaneous hyaluronosis in shar pei dogs.

Animals: 10 healthy shar peis and 10 healthy beagles used as controls.

Procedures: Ultrasonographic examination of the skin was performed on 4 cutaneous sites using a 13-MHz linear-array transducer, and the mean value of 3 measurements was calculated. Ultrasound results were also compared with histological findings of skin specimens stained with H&E, Alcian Blue at pH 2.5 and Masson's trichrome stains and with histometric measurements of cutaneous skin thickness using a Leica MZ FLIII microscope. Finally, ultrasonographic results were statistically related to age and gender of selected animals and to plica thickness measurements performed from the same area of ultrasound examination by a Trimmeter digital skin fold meter instrument.

Results: A clear correspondence was demonstrated between ultrasound results and histological and histometric analysis in both groups of animals. In shar peis, no correlation was found between ultrasound results and age and gender whereas in beagles, a weak positive correlation was found only between dorsal neck and frontal regions and age. Moreover, on the total of results, a positive correlation was found in shar peis between ultrasound measurements and plicometer results.

Conclusions and Clinical Relevance: Ultrasound is a useful tool to assess multiple cutaneous parameters and in shar pei dogs it may be considered as a valid alternative to other invasive methods such as histology, to objectively estimate the degree of hereditary cutaneous hyaluronosis.

Introduction

Shar pei dogs are distinguished from other breeds by their characteristic skin folds, a feature recently demonstrated to be hereditary in origin.[1] Tight thick wrinkles are usually numerous in puppies but tend to decrease in adults. Indeed, such as documented in a comparative human disorder associated with generalized folding and thickening of the skin, it may be reasonable to speculate that the normal age-related decline in ratio of body surface area to weight could be the underlying mechanism for the changes in wrinkling noted with the advance of growth.[2]

This unusual wrinkling and thickening of the skin has been considered to be the consequence of an abnormal deposition of mucin, and therefore the disease entity is found in text books under the name of cutaneous mucinosis. Recently, by means of histochemistry and an *in vitro* model, the main component of mucin substance has been demonstrated to be hyaluronic acid (HA),[3,4] a large unsulfated glycosaminoglycan that once synthesized is extruded out of fibroblast cells where it contributes to the assembly and organization of the extracellular matrix. Therefore, the term hereditary cutaneous hyaluronosis (HCH) seems to describe this condition more accurately.

Nevertheless, to the best of our knowledge, no studies are available in shar pei dogs to assess the degree of HA deposition and the relative increase in skin thickness in an objective and non-invasive manner. For example in humans, ultrasonography is considered a versatile, painless, low-risk and non-invasive procedure that provides real-time visual information about skin thickness and skin density. Indeed, it has been extensively used in inflammatory disorders characterized by thickening and fibrosis of skin such as sytemic sclerosis,[5,6] in pre-operative tumor thickness measurements[7,8] and in the evaluation of the age-related changes of skin thickness.[9] Moreover, in cutaneous sclerosis, several other mechanical instruments such as elastometer, twistometer, cutometer and plicometer, have been also used to exert a controlled physical force on the skin and to better assess cutaneous involvement.[10]

In veterinary medicine, ultrasonography has been also used to characterize benign and malignant skin tumors,[11] to evaluate skin thickness[12] and the changes of skin thickness in relation to hydration status and fluid distribution in dogs.[13] Instead, the plicometer instrument has found application to examine the dietary effects on skin biophysical parameters.[14]

According to this background, the objectives of this study were to demonstrate if the ultrasound imaging system could be a useful instrument to reflect the excessive HA dermal deposition in shar pei dogs. First, we compared ultrasonographic results with histological findings and histometric measurements of skin samples obtained from the same selected regions of ultrasonographic examination. Secondly, the investigation sought to correlate ultrasonographic results with age and gender of selected animals, in order to understand if these parameters could affect skin thickness. Finally, to further assess cutaneous involvement, our last objective was to correlate skin fold measurements obtained by plicometer instrument with ultrasound results.

Materials and Methods

Study population

The study was perfomed with 20 dogs, of which 10 were shar peis coming from two shar pei breeders in Catalunya (Spain), and 10 were beagles, used as controls and coming from the blood bank of the Veterinary Faculty of Universitat Autònoma de Barcelona (UAB) and from the kennel of beagles of UNIVET.[a] Shar pei group included 6 sexually intact adult females and 4 adult intact males, ranging from 1 to 3 years of age (mean ± SD, 1.8 ± 0.9) and from 16 to 22 kg (mean ± SD, 18.4 ± 2.4) (Table 1). On clinical examination, all of them were considered affected by HCH of variable severity. Nine shar peis were phenotipically classified as in conformity with the American standard of the breed, characterized by pronounced loose skin covering the forehead, and extending to the neck, withers and hind legs (Figure 1A). Only 1 shar pei was classified as in conformity with the more traditional Chinese standard, characterized by tight wrinkles covering only the withers and the forehead (Figure 1B). The beagle group included 7 sexually intact adult females and 3 males, ranging from 3 to 9 years of age (mean ± SD, 5.8 ± 2.4) and from 8 to 14 kg (mean ± SD, 11.1 ± 2.6). All dogs were considered clinically healthy on the basis of normal results of clinical examination, complete blood count and total protein analysis. A routine urine analysis was also performed in each dog.

All procedures outlined in this project were approved by the breeders of shar pei dogs and by the Ethical Committee of the UAB, prior to initiation.

Ultrasound measurements of the skin

A B-mode real-time ultrasonographic examination of the skin thickness was performed with an Esaote ultrasound machine (MyLab® 70)[b] fitted with a linear high-frequency (13 MHz) transducer. Four regions (frontal, dorsal neck, sacral and left metatarsal regions) were selected. After hair clipping, skin was gently cleaned with ethanol alcohol to remove any cutaneous surface residues, and an abundant amount of acoustic gel[c] was applied between the skin surface and the transducer in order to focus the skin on the focal zone of the probe. One experienced investigator (YE) performed ultrasonographic examination by placing the transducer perpendicularly to the skin, and the following order of skin examination was adopted: frontal region was first examined halfway along the line connecting the rostral margins of the supraorbital processes; the dorsal neck region was then examined at the junction between the second and third cervical vertebrae, whereas the sacral region was subsequently evaluated halfway along the line connecting the left and the right tuber coxae. For all these regions, animals were maintained in sternal recumbency. The dorsal surface of the metatarsus of the left hind limb was examined after positioning the animal in right lateral recumbency. Once identified the surface epidermis, epidermis-dermis and dermis-subcutis interfaces, only the “full” skin thickness (a composite of epidermis and dermis) was measured by the electronic calliper of the ultrasound machine. Three measurements were performed each one at a distance of about 5 mm and the time-gain compensation was maintained at 78%. Enlarged images of a width 30 mm and depth of 22 mm were evaluated.

Plicometer examination

At the same selected areas of ultrasound examination, skin fold measurements were carried out in triplicate using a Trimmeter digital skin fold meter with a digital display[d] and capable of measuring from 1 to 100 mm. An assistant pinched the skin between the thumb and forefinger and one of the investigators (GZ) gently placed the left hand arm of the instrument first, and then the right hand arm provided with sensor into contact with skin fold. After the closure of the arms of the instrument, an audible signal indicated that the measurement has been taken. Taking the nearly compression-free ultrasound measurements as the standard of a composite of epidermal plus dermal thickness, skin fold thickness measures were considered as the result of a double layer of skin together with underlying fatty tissue.

Histological examination

Skin samples from each one of the four ultrasonographic examination points were taken using a 6-8 mm punch biopsy or excisional biopsy, immediately fixed in buffered 10% formalin solution for 24 hours, and paraffin embedded. Sections were then stained for histological examination with Haematoxylin and Eosin (H&E), Alcian Blue at pH 2.5 and Masson's trichrome stains. Analysis of skin thickness on H&E stained samples was performed using a Leica MZ FLIII microscope[e] equipped with a Leica DC500 CCD camera[f]. The images were then collected using an Universal Imaging MetaMorph software[g] and histometric measurements were performed.

Statistical analysis

Statistical analysis was performed with a commercial statistical software.[h] A one-way ANOVA test was used to value hypotheses about differences in the average values of skin thickness between shar peis and beagles after ultrasound and plicometer measurements, respectively. Differences in each selected region, were instead calculated using a Tukey-Kramer Multiple Comparisons Test. In all tests, statistical significance was set to a P value <0.05. A Pearson correlation test and a regression analysis were used to value the relationship between ultrasound measurements and age, gender, and plicometer results. A P value was computed on T-test results.

Results

Ultrasound measurements

In accordance with previous studies,[12] three distinct layers were observed ultrasonographically in both shar pei and beagle dogs: first, a hyperechoic well-defined superficial layer at the interface between the coupling gel and the skin corresponding to epidermal entry echo. Under this, a less echogenic layer of varying intensity corresponding to the epidermis-dermis, and a deep layer corresponding to subcutaneous tissue and containing thin linear hyperechoic areas on a nonhomogeneous hypoechoic pattern. In shar peis, a hyperechogenicity with a fine and granular echotexture was observed in the dermis of all dogs of American line (Figure 2A) whereas in the

dog of Chinese line the dermis was more densely hyperechogenic (Figure 2B). A double-layered appearance of the dermis, with the superficial layer more echogenic than the deeper layer, was instead detected in 6 out of 10 beagles (Figure 2C).

In shar peis, the mean skin thickness ± SD was of 4.10 ± 56 mm against 2.23 ± 41 mm of beagles, and it was greatest at sacral region followed by dorsal neck, frontal and metatarsal regions. In beagles, skin thickness was greatest at metatarsal, followed by dorsal neck, frontal and sacral regions. A significant difference between shar peis and beagles was demonstrated in the sacral, dorsal neck and frontal regions ($P<0.001$), whereas in the metatarsal region this difference was also significative but less pronounced ($P<0.01$) (Figure 3). Nevertheless, by multiple comparison test no significant differences in skin thickness from examined body regions were detected in both breeds. In shar peis, no correlation was established between ultrasound results at single body regions and age and gender, whereas in beagles, a positive correlation was shown between age and ultrasound results at dorsal neck (r, 0.666; $P=0.036$) and frontal (r, 0.750; $P=0.012$) regions, but no correlation was established when ultrasound results were correlated with gender.

Plicometer measurements

Three measurements of cutaneous plica thickness derived from plicometer skin fold meter instrument application, were made in all shar peis of American line (Figure 4A), in the shar pei of Chinese line (Figure 4B) and in beagles (Figure 4C). The media ± SD was demonstrated to be of 7.55 ± 2.04 mm in shar peis against 6.22 ± 2.72 mm of beagles.

Among the examined cutaneous regions, the largest plica thickness was evident in the sacral region followed by the dorsal neck, frontal and metatarsal regions in shar peis, whereas in beagles dorsal neck region was the most thickened, followed by the sacral, frontal and metatarsal regions (Figure 5). A significant positive difference between shar peis and beagles was detected only at the frontal region with a $P<0.001$, and at the metatarsal regions with a $P<0.05$. By multiple comparison test, significant differences ($P<0.001$) in plica thickness were instead detected in both breeds especially between the sacral and frontal region and the sacral and metatarsal region. In shar peis, a weak positive correlation was found between ultrasound and skinfold caliper measurements when statistical results referred to each single region, but this correlation was clearly positive on the total of results (r, 0.538; $P=0.0038$) (Figure 6). On the contrary, in beagles the correlation between ultrasound and plicometer results showed to be significantly positive at each single region but no significative on the total of results (r, 0.167; $P=0.302$).

Histologic examination and histometric measurements

In shar peis of American line, dermal collagen fibers were demonstrated to be separated by a diffuse pale grey-pink substance corresponding to mucinous material (Figure 7A). In the shar pei of Chinese line, scarce mucinous material was detected between collagen fibers (Figure 7B), whereas in control dogs this material was not found (Figure 7C).
Histometric measurements of skin thickness were then performed from each selected area, and when compared with ultrasonographic results a clear correspondence was shown. Skin thickness

was greatest in shar peis of American line, followed by shar pei from Chinese line and beagles. All these findings are illustrated in the Figure 7.

By Alcian Blue stain at pH 2.5, in the dermis of shar pei dogs collagen fibers were scattered between a network of basophilic material, considered to be a characteristic aspect of acid glycosaminoglycans such as HA. This finding was clearly demonstrated in shar peis of American line (Figure 8A), whereas in shar pei of Chinese line it was less evident (Figure 8B). In control dogs this material was poorly detected in some skin samples (Figure 8C).

By Masson stain, collagen fibers stained of an intense turquoise material that in shar peis of American line showed to be less densely distributed (Figure 8D) in comparison with shar pei of Chinese line (Figure 8E) and control dogs (Figure 8F).

Discussion

The challenge of this study was to point out the efficiency and reliability of ultrasonographic technique as a new non-invasive method of investigation and quantification of different degrees of cutaneous hyaluronosis in shar pei dogs. In fact, in this breed the diagnosis of cutaneous hyaluronosis has long relied only on clinical observation and palpation eventually supported by histological findings.[3,15] Nevertheless, with the recent established diagnostic role of ultrasounds technique to evaluate the appearance of normal canine skin[12] and the changes of canine skin thickness in relation to hydration status and fluid distribution,[13] a new frontier has been opened in veterinary dermatology.

The technique of ultrasonography involves the detection of reflected sound waves through tissues that possess inherently different acoustic properties. In particular, echoes in the dermis are the result of the reflection of the ultrasound waves at the boundaries between dermis components, as collagenous and reticular fibers, dermal ground substance, sebaceous and sweat glands and the surrounding water-rich ground substance.[16] For example, a decrease of the echogenicity of the dermis may be due to the excess of the fluid in the *interstitium* separating the collagen fibers with consequent distension of the fiber network.[17] Therefore, the resulting ultrasound image that is produced consists of regions of varying echogenicity, which correlate to different histological regions of the skin.[18]

Furthermore, selection of the appropriate transducer is an important aspect of soft-tissue bedside ultrasonography. Indeed in humans, 20 MHz scanners are used for measuring skin thickness and assessing inflammatory skin disorders, whereas 7.5-15 MHz sounds are mostly used in dermatologic oncology.[17]

In accordance with Diana *et al.*,[12,13] a 13-MHz linear array transducer was used in this study, and assessments of appearance were possible as well as observation of the skin layers below the surface in all four selected areas. In fact, in both shar pei and beagle dogs, a hyperechoic well-defined superficial layer at the interface between the coupling gel and the skin was first observed. Its echogenicity was considered to depend on the thickness of the *stratum corneum* and the amount of air trapped between the keratotic scales. A second less echogenic and thicker layer corresponding to the remaining epidermal layers plus dermis was then detected, followed by a third hypoechoic layer interspersed with hyperechoic linear areas running mostly parallel to the skin and representing subcutaneous tissue. Nevertheless, in shar peis of American line the second layer

showed a fine and granular hyperechogenic texture in all selected body regions in comparison with the shar pei of Chinese line, in which an overall hyperechogenic and homogeneously spread second layer was observed. On the contrary, echoes of various intensities changing from low to high echogenic pattern were variably observed at dermal level in more than the half of beagles. In the last case, the variability of the ultrasonographic pattern was probably related to the differing amount of dermal fluid storage in the *interstitium*, as previously demonstrated,[13] whereas the different hyperechogenicity and texture observed in shar peis was likely related to the HA deposition and to the ability of this molecule to stabilize intercellular structures *via* the formation of a viscoelastic network together with collagen and elastic fibers.

The accuracy of ultrasonography was further corroborated by histological results. Indeed, in shar peis of American line HA showed to spread widely between collagen fibers normally arranged, whereas in the dermis of the shar pei of Chinese line HA distributed in a small amount and it was poorly detected in the dermis of controls such as demonstrated by Alcian blue stain.

Finally, a clear correspondence was also found between histometric measurements of skin samples stained with H&E and ultrasound skin thickness evaluation. Indeed, shar peis of American standard that are known for their abundant bundles of wrinkles, showed to have a higher skin thickness when compared with the shar pei of Chinese standard known for less skin folding distribution and with beagles, in which wrinkles are not normally present. It means that a part of intra-breed, systematic regional variations of skin thickness may be also dependent on inter-breed differences. In general in dogs, skin thickness has been demonstrated to decrease dorsally to ventrally on the trunk and proximally to distally on the limbs, with the thickest skin located over the head, dorsum of the neck, back and sacrum.[19]

Ultrasonographic results of this study demonstrated that while skin thickness in shar peis was greater in the sacral area followed by dorsal neck, frontal and metatarsal regions, in beagles the thickest region was the metatarsal, followed by dorsal neck, frontal and sacral regions. Therefore, could be hypothesized that many other factors have an influence on the variable rate of skin thickness in different body regions. For example in humans, skin thickness has been demonstrated to be under the control of hormonal factors such as sex estrogens which increase dermal hydroscopic properties probably through enhanced synthesis of dermal HA,[20-22] and aging which modifies the viscoelastic properties of skin by increasing the breakdown of extracellular matrix components.[23,24]

As consequence, in our study population we investigated if a correlation could exist between gender and dermal thickness and if a decreased glycosaminoglycan content with consequent skin atrophy would occur in older individuals. Contrarily to our expectations, no correlation was found in both shar peis and beagles between ultrasound measured skin thickness and gender, whereas only in beagles a weak correlation was detected between ultrasound results at dorsal neck and frontal regions and age. Although this study was conducted with only two breeds to minimize inter and intra-individual variability, failure to find a correlation could have depended on the small number of selected individuals and on variability in mean age between the two groups. In the future, more correlated results could be benefited by using a larger study population and by the standardization of hormonal status in homogeneous groups of aged animals.

Ultrasound has been also used in dogs to measure subcutaneous fat and to predict the total body fat.[25,26] However, in this study and as previously reported,[12] a clear demarcation of the distal subcutaneous tissue boundary useful to make measurements, was not obtained in the ultrasound

images. This absence, could depend on reliable imaging given by the transducer of the subcutaneous tissue, structure that is known to project into the overlying dermis as papillae adiposae that surround hair follicles, sweat glands and vasculature, and to form attachments to the underlying fibrous skeletal components.[19] To obviate this limitation, in this study was introduced the plicometer, an instrument that in humans is typically used for the assessment of nutritional status in cross-sectional studies as well as in follow-ups of medical conditions such as growth hormone replacement therapy and obesity.[27,28] The majority of data involving body composition assessments deal with the correlation of skinfold thickness to percent body fat and are based on prediction equations based on mathematical relationships.

In both groups of animals, we limited our study to the simple measurement of skin thickness in triplicate. Despite a high internal variability of the results from each region, in shar peis a positive correlation was found between ultrasound and skinfold caliper measurements on the total of results. On the opposite, beagles showed a low internal variability but a high variability on the total of results that was reflected on the coefficient of correlation. Our analysis suggested that this variability of correlation between ultrasound and skinfold caliper derived results could be the consequence of changes in the compressibility of subcutaneous tissues associated with breed, age and nutritional status. Therefore, given the small size of our study population and the paucity of comparable data concerning the distribution of adipose tissue in dogs, in the future are necessary further studies that better define the body region whereas less variability exists and that therefore could warrant a more specific correlation with ultrasound results.

In conclusion, our work demonstrated that diagnostic non-invasive tool such as ultrasound could be used to assess multiple cutaneous parameters and that it could be easily used as a valid alternative to other more invasive methods of investigation such as histology to objectively estimate in shar pei dogs the degree of hereditary cutaneous hyaluronosis.

Aknowledgements

The authors thank Dr. Vito D'Agostino (Science of Information, Statistical Department of Puglia Region, Italy) for his statistical support, shar pei breeders Rosa Bellés and Nuria Verdés, for their precious collaboration and Dr. Rafael Ruiz de Gopegui and Dr. Anna Puigdemont for giving us the possibility to include in our study population beagles from the blood bank of the Veterinary Faculty of UAB and UNIVET, respectively.

TABLES and FIGURES

Shar peis				Beagles			
Dog	Age (y)	Weight (kg)	Sex	Dog	Age (y)	Weight (Kg)	Sex
1	1	21	F	1	3	8	F
2	3	19.1	F	2	3	11	M
3	3	19	M	3	7	12.1	F
4	1	22.2	M	4	9	14.1	F
5	1	16	F	5	8	14.9	M
6	1	21	F	6	9	10.1	F
7	1	15.7	M	7	3	11	F
8	3	16	M	8	4	14	M
9	1	18	F	9	6	8	F
10	2	16.3	F	10	6	8	F
Mean ± SD (Age)		1.8 ± 0.92		Mean ± SD (Age)		5.8 ± 2.44	
Mean ± SD (Weight)		18.43 ± 2.41		Mean ± SD (Weight)		11.12 ± 2.64	

Table 1. Study population. Reported age, weight and sex of both shar pei and beagle dogs and mean ± Standard Deviation of age and weight.

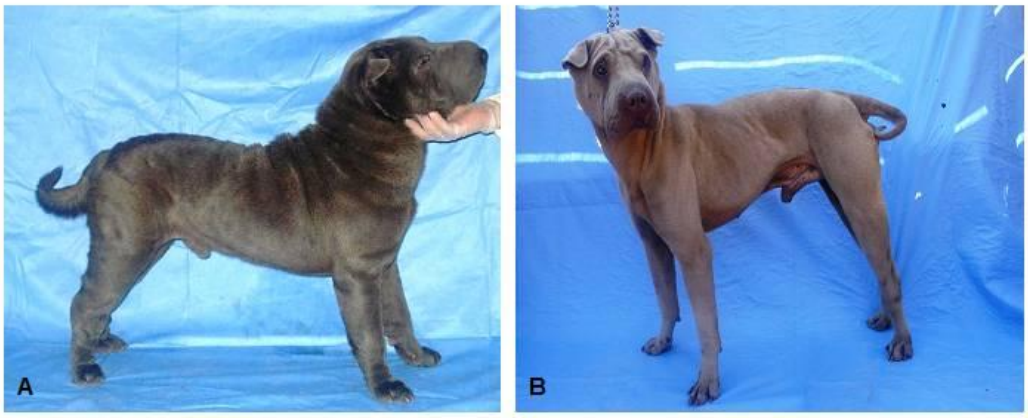

Figure 1. Standard of shar pei breed. American standard: overall appearance of a robust and well-muscled dog, with broad muzzle (hippopotamus muzzle) and abundant bundles of wrinkles on the head, neck and withers (A). Chinese standard: overall appearance of a strong, compact dog with narrowed muzzle and tight wrinkles on the forehead and withers (B).

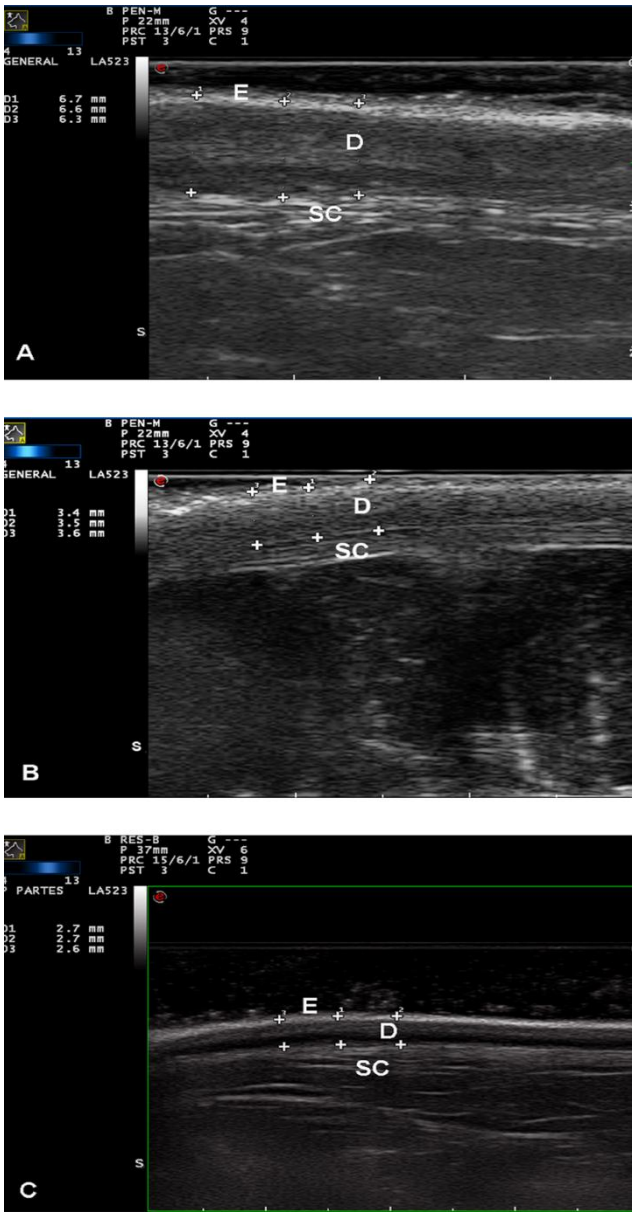

Figure 2. Ultrasonographic appearance of the skin and measurements of skin thickness on sacral region (S) in shar peis and control. Detection of three distinct layers: a hyperechoic well defined superficial layer corresponding to epidermal entral echo (E), a less echogenic layer corresponding to epidermis plus dermis (D) and a deeply layer containing linear hyperechoic images corresponding to subcutaneous tissue (SC). A fine and granular hyperechogenic texture is observed in the dermis of shar pei of American standard (A). The hyperechogenicity is homogeneous in the dermis of Chinese standard (B). Two distinct bands of different echogenicity are observed in the dermis of control (C). Three measurements of skin thickness (D1, D2, D3) corresponding to epidermis plus dermis are indicated on the left of each image. Shar pei of American standard that has been selected for this figure, represents the dog with the highest skin thickness within the group of shar peis.

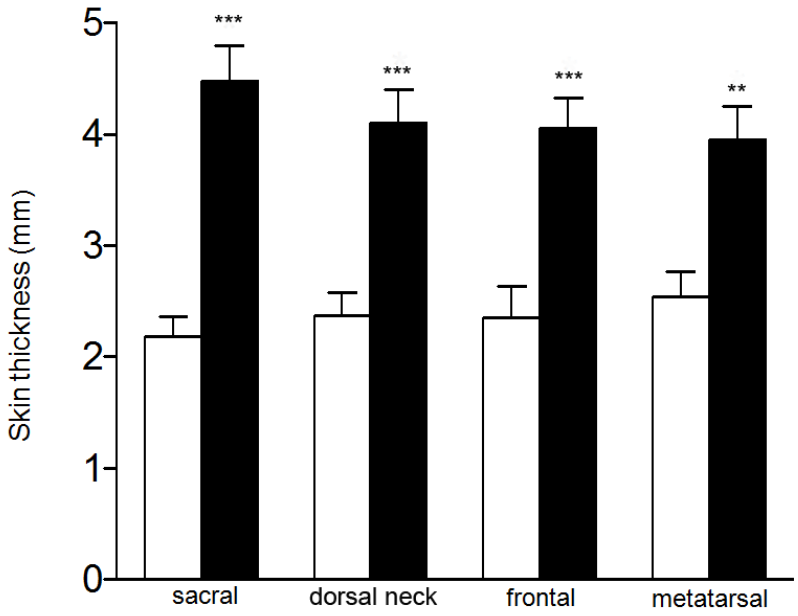

Figure 3. **Comparisons of ultrasound skin thickness measurements between shar peis (black columns) and beagles (white columns) at 4 body regions (sacral, dorsal neck, frontal, metatarsal).** ****P* value <0.001, ***P* value <0.01.

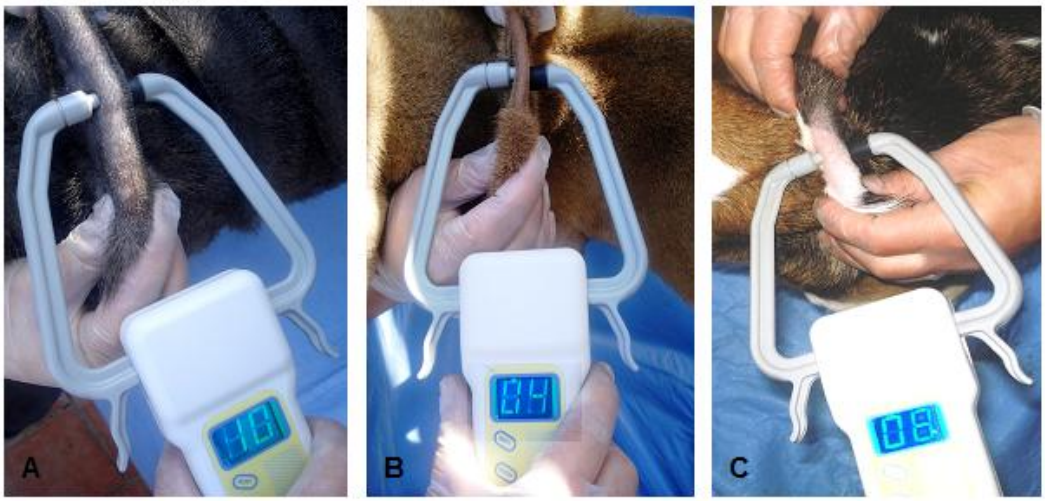

Figure 4. Cutaneous plica thickness measured by plicometer instrument applied on dorsal neck region. The higher plica thickness is in the shar pei of American standard (A). The lower plica thickness is in the shar pei of Chinese standard (B). Also in the beagle, plica thickness is high in the dorsal neck region (C).

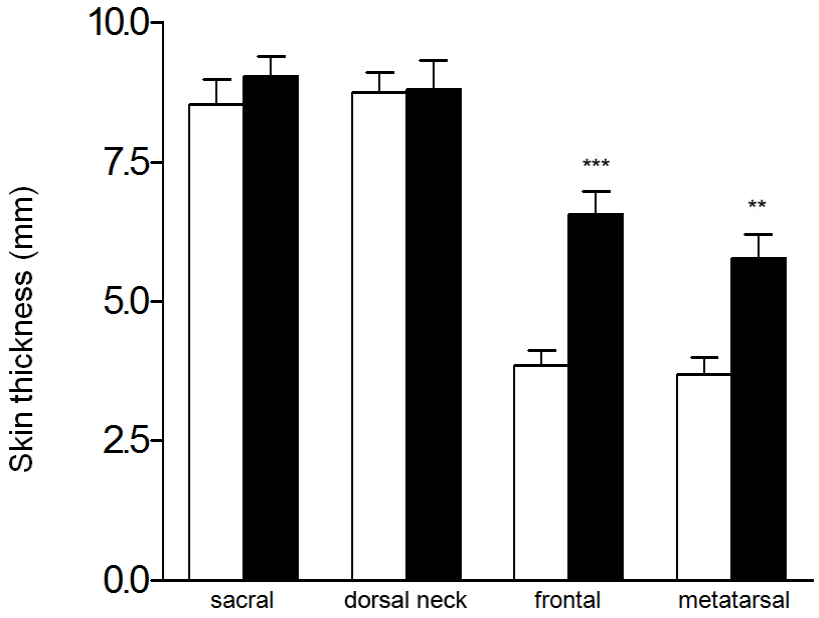

Figure 5. Comparisons of plicometer skin thickness measurements between shar peis (black columns) and beagles (white columns) at 4 body regions (sacral, dorsal neck, frontal, metatarsal). ****P* value <0.001, ***P* value <0.01.

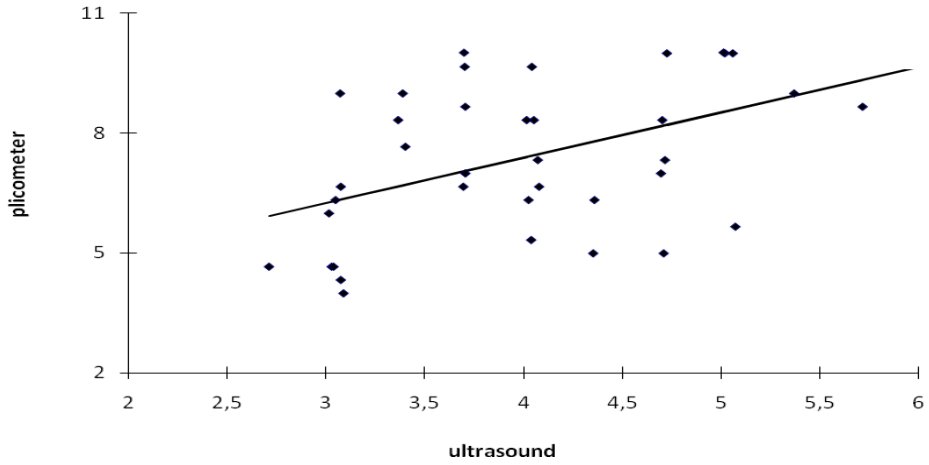

Figure 6. Correlation by ultrasound results (mm) and plicometer results (mm) in shar pei dogs. A positive correlation is demonstrated (*r*, 0.538; *P*=0.0003).

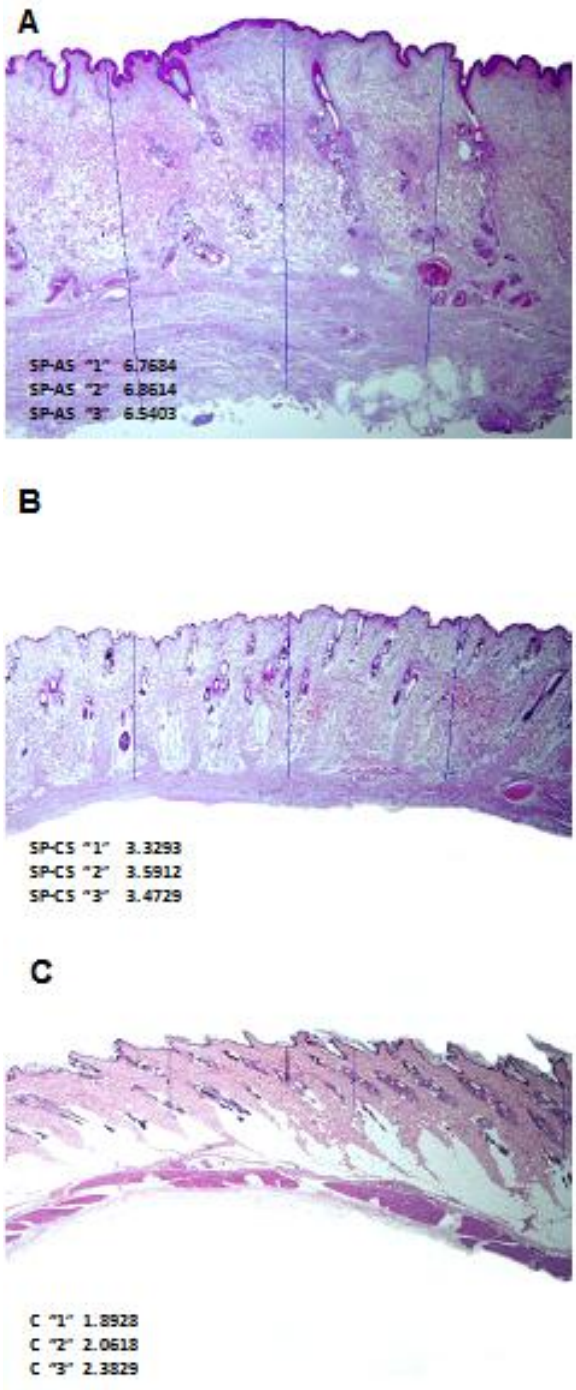

Figure 7. Skin sections of shar peis and beagle from frontal region. With H&E staining, presence of a diffuse pale, pink substance in the dermis of shar pei of American standard (SP-AS) (A). This finding is less evident in the shar pei of Chinese standard (SP-CS) (B) and not evident in the beagle (C). On the left of each image are reported three measurements (“1”, “2”, “3”) of the skin thickness performed by Leica microscope. Shar pei of American standard that has been selected for this figure, represents the dog with the highest skin thickness within the group of shar peis.

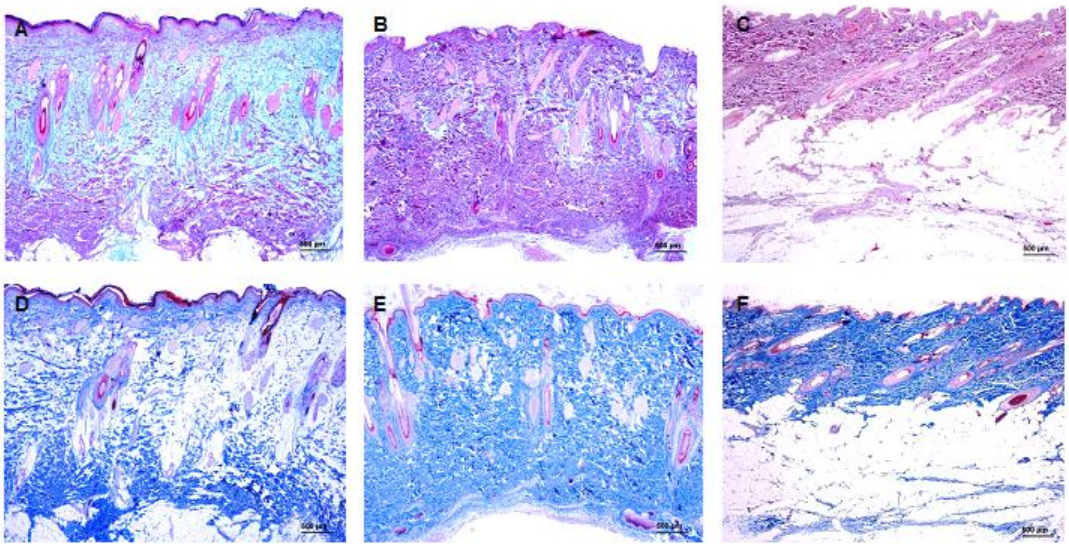

Figure 8. **Skin sections of shar peis and beagle from dorsal neck region.** By Alcian Blue stain, in the dermis of shar pei dog of American standard collagen fibers are scattered between a network of basophilic material corresponding to HA (A). This finding is less evident in the shar pei of Chinese standard (B) and in the beagle (C). By Masson stain, in the shar pei of American standard collagen fibers stained of an intense turquoise, are less densely distributed (D) in comparison with shar pei of Chinese standard (E) and beagle (F).

Footnotes

[a] UNIVET, Veterinary Diagnostic Service Ltd, Barcelona, Spain.
[b]Esaote ultrasound machine (MyLab® 70), Esaote España S.A., Barcelona, Spain.
[c]Polaris II ultrasound gel, GE Medical Systems, España S.A.
[d]Trimmeter digital skinfold, Robert Pringle Engineers Ltd Harlow, Essex, UK.
[e]Leica MZ FLIII, Leica Microsystems, Wetzlar, Germany.
[f]Leica DC500 CCD camera, Leica Microsystems, Wetzlar, Germany.
[g]Universal Imaging MetaMorph v.5.1 software, Universal Imaging Corporation, Downington, PA, USA.
[h]GraphPad InStat 3.05, GraphPad Software, Inc., San Diego, CA, USA.

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