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1 **Ultrasound image processing to estimate the structural and functional**
2 **properties of mouse skeletal muscle**

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24 **ABSTRACT**

25

26 Noninvasive imaging techniques are increasingly used for monitoring muscle behavior in mice.
27 However, muscle is a complex tissue that exhibits different properties under passive and active
28 conditions. In addition to structural properties, it is also important to analyze functional
29 characteristics. At present, such information can be obtained with ultrasound elastography.
30 However, this technique is poorly used for small rodent models (mice and gerbils). Thus, this study
31 aims at establish referent hindlimb muscle data, and experimental guidelines, for wild-type (WT)
32 control mice as well as the TIEG1 knockout (KO) mouse model that is known to exhibit skeletal
33 muscle defects.

34 Ultrasound was performed with the Aixplorer machine using a SLH 20-6 linear transducer probe
35 (2.8 cm footprint). A region of interest (ROI) was placed around a superficial group of muscles.
36 Subsequently, from the B-mode image, a classification of all the muscles and ultrasound biomarkers
37 such as echo intensity and texture anisotropy have been determined. The influence of the gain
38 setting (from 40% to 70%) was analyzed on these parameters. Moreover, the elasticity (E) was also
39 measured within the ROI.

40 This study provides a suitable methodology for collecting experimental data: 1) the correct range of
41 gain (between 50% and 70%) to apply for the ultrasound measurement of muscle structure, 2) the
42 structural and functional referent data for a group of healthy muscles, 3) the gray scale index, the
43 texture anisotropy and the elasticity ($E_{TIEG1_KO}=36.1 \pm 10.3\text{kPa}$, $E_{WT}=44.4 \pm 13.4\text{kPa}$) parameters,
44 which were obtained for a group of muscles as a function of genotype.

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49 1. Introduction

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51 The functional properties of animal muscle can be characterized *in vitro* using mechanical tests
52 (stretch, relaxation, indentation, etc...) to measure their passive and active mechanical properties
53 [1] or *in vivo* using non-invasive techniques such as imaging devices or surface electromyography
54 [2] to elucidate their physiological activity. Magnetic Resonance Imaging (MRI) has been
55 extensively performed on rodent muscle to analyze the physiological composition (percentage of fat
56 and water, presence of fibrosis, texture...) and muscle metabolism (creatine, choline, lipid ...) through spectroscopy acquisitions. MRI can also reveal the anisotropic muscle behavior using a
57 diffusion tensor imaging sequence. While MRI offers extensive information about the muscle
58 structural properties, the quantification of the functional properties relating to elasticity cannot be
59 determined by this imaging technique.
60

61 More recently, quantitative muscle ultrasound shear wave elastography (SWE), performed with
62 the Aixplorer machine, has been reported to be an effective technique for the non-invasive
63 assessment of the functional properties of human skeletal muscle [3,4]. Additionally, another group
64 has used the Aixplorer machine to examine the correlation between the elastic properties and
65 pathological characteristics of spinal cord injuries in a rat model [5]. However, in the literature,
66 there is an important lack of studies using elastography techniques on small rodents (such as mice
67 and gerbils). Recently, we have developed an SWE experimental protocol to characterize the *in vivo*
68 elastic properties of the musculotendinous system of healthy mice. In order to further develop the
69 use of the Aixplorer machine for these purposes, it is also necessary to establish referent data with
70 regard to structural properties and echogenicity of muscle. For that, we analyzed the influence of
71 the setting parameter (different gains) on ultrasound biomarkers (echo intensity, texture anisotropy)
72 which were determined for both healthy and dystrophic mouse hindlimb muscle.

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76 **2. Materials and methods**

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78 **2.1. Mice and Study design**

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80 Eight wild-type (WT) and eight TIEG1 (TGF β Inducible Early Gene-1) knockout (KO) mice were
81 used. The generation of TIEG1 KO mice has been previously described [6] and this model has been
82 chosen for its known morphological (hypertrophy, muscle disorganization, ...) changes and
83 functional defects (lower elasticity, etc...) in hindlimb muscle compared to WT littermates [1,7,8].
84 All mice were maintained in a temperature controlled room ($22 \pm 2^\circ\text{C}$) with light/dark cycle of 12
85 hours. Animals had free access to water and were fed with standard laboratory chow ad libitum.
86 This study was carried out in strict accordance with the recommendations in the Guide for the Care
87 and Use of Laboratory Animals of the National Institutes of Health (Permit Number: DUO-4776).

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89 **2.2. Shear Wave Elastography (SWE) imaging**

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91 All mice were anesthetized with 1.5% isoflurane and a mixture of O₂/air (1:1) at an output of 0.7
92 L/min and placed in a supine position.

93 Landmarks were defined to place the mice and the paw in a reproducible manner. Muscles in the
94 right hindlimb were imaged by the same operator with an ultrasound (US) machine (Aixplorer
95 MultiwaveTM System, Supersonic Imagine, Aix-en-Provence, France) using a novel SLH 20-6
96 linear transducer probe (2.38 cm footprint, 192 composite elements, effective bandwidth from 6 to
97 20 MHz) (Fig. 1A).

98 The probe was placed on the surface of the hindlimb, which was shaved, parallel to the Achilles
99 tendon and aligned with respect to muscle fiber orientation. To obtain ideal acoustic impedance a
100 thin layer of acoustic gel was applied. To ensure that the probe was reproducibly placed, a visual
101 control was performed on the B-mode (anatomical) image (Fig. 1B) where the heel and the bones
102 (fibula and tibia) surrounded by the group of muscles are present in each ultrasound (US)
103 acquisition for all mice.

104 To identify the muscles present in the B mode image, an acupuncture needle (0.25 x 25mm) was
105 inserted within the superficial hindlimb muscles (Fig. 1B). Subsequently, a dissection of the seven
106 muscles (Fig. 1C) was performed to allow for the superimposition of the names of the muscles with
107 the corresponding B-mode image.

108 The Aixplorer Multiwave™ System generates two types of waves that propagate within the tissue: a
109 compression wave that creates a high-quality B-mode image showing the anatomical structure
110 within the hindlimb (skin, muscle, tendon, bone), and a shear wave that provides a quantitative
111 color-coded map (rectangular box : 1 cm x 1.5 cm) of tissue elasticity (Fig. 1D). To obtain mapping
112 of elasticity with the shear wave elastography (SWE) sequence, the setting parameters were:
113 musculoskeletal preset, resolution mode enabled, tissue tuner at 1540 m/s, pulse duration: 600 μs,
114 dynamic range at 60 dB, and lateral / vertical resolution of 140 μm / 205 μm. The depth setting was
115 fixed at 2 cm for all mice during examination to display the entire muscle. The focus range was set
116 between 0.5 cm and 1 cm depth for all mice. The setting parameters for the B-mode were:
117 musculoskeletal preset, pulse repetition frequency: 20 kHz, spatial resolution: 38 μm, super
118 compound disabled, harmonic disabled penetration and HD mode. The gain can be increased or
119 decreased as a function of the depth of the explored area. Thus, to analyze the influence of these
120 parameters on the structural properties, four different gains (40%, 50%, 60% and 70%) were tested.
121 Time-gain compensation was maintained in the same position for all depths.

122 The SWE mode is based on the generation of localized acoustic radiation force by the probe which
123 generates a propagation of transversal waves. Assuming that the ZM muscle was linearly elastic,
124 isotropic, homogeneous and incompressible, the range of elasticity (Young's modulus : E) was set
125 between 0 kPa and 180 kPa, which corresponds to a shear wave velocity (V) range of 0 - 7.7 m/s
126 using the equation $E = 3 \rho V^2$ where ρ is the density of muscle (1000 kg/m³) [10].

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2.3. Image analysis

In the B-mode image, the muscle group in the mouse hindlimb was visualized and a region of interest (ROI) was manually drawn around the muscle tissue composed of three specific muscles (PB (#5): peroneus brevis, Sol (#6): soleus and Gas (#7): gastrocnemius). To quantify structural and SWE parameters, a semiautomatic method was developed using ImageJ 1.46/Java 8 software (National Institute of Health, Bethesda, MD, United States) [9]. Subsequently, the ROI was superimposed to the elasticity image (Fig. 1D) and by using the ROI Manager Tool of ImageJ the following parameters were measured: mean, standard deviation (SD) and coefficient of variation (i.e ratio of standard deviation to mean) for echo intensity (EI) and mean elasticity (E: Young's modulus) parameters (Fig. 2). From the mean of echo intensity, which the gray value varies from 0 to 255, a gray-scale index (GSI) was defined as:

$$\text{GSI} = 1 - \frac{1}{255 \cdot N} \sum_{i=1}^N \text{EI}_i$$

with EI_i the echo intensity of pixel i in the selected area [10]. When GSI is close to 1 or in contrary close to 0, the B-mode image changes from dark to clear, respectively.

The ROI of B-mode images was also analyzed for texture analysis by computing the gradient of the image and the local covariance matrix C using ImageJ [9]. The 2x2 covariance matrix C was computed for each pixel and evaluated from local intensity variations around each pixel in a box of 5x5 pixels. The two eigenvalues λ of C were retrieved and sorted with $\lambda_1 > \lambda_2$. A texture anisotropy index (TAI) was computed for each ROI and defined as the mean value of the texture image $\alpha = 1 - \lambda_2 / \lambda_1$. Therefore, a TAI value close to 1 indicates the strong predominance of one orientation in the ROI (i.e., strong local texture anisotropy) whereas TAI close to 0 indicates a diffuse image with no predominant orientation (i.e., weak local texture anisotropy).

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2.4. Statistical analysis

The XLSTAT™ software package was used to perform all statistical analyses. All parameters were expressed as the mean \pm standard deviation. Nonparametric two-sample Kolmogorov-Smirnov tests were performed in order to compare the elasticity values (E) and the B-mode values (GSI, TAI) between WT and TIEG1 KO mice. The statistical analysis was considered significant for $p < 0.05$.

3. Results and discussion

3.1. Identification of hindlimb muscle

Ultrasound acquisition, as opposed to MRI imaging, did not allow for individual visualization of all of the hindlimb muscles of mice. In the present study, the ultrasound B-mode image revealed three distinct groups of muscles (Group 1 composed of peroneus brevis (#5), soleus (#6) and gastrocnemius (#7); Group 2 composed of plantaris (#3) and peroneus longus (#4) and Group 3 composed of the tibialis anterior (#1) and extensor digitorum longus (#2)). Noninvasive imaging techniques are increasingly used for monitoring muscle behavior of mice. Thus, the present classification, which is quite rare in the literature, could allow for the use of an optimal ultrasound methodology to build a large database of muscle structure and function before or after a given treatment or intervention. In addition, this protocol will enable the development of a very precise automated method for the follow-up of target muscles composed specifically of slow twitch fibers such as the soleus or fast twitch fibers such as the EDL.

3.2. Influence of gain settings on echogenicity and texture anisotropy

To accurately establish the ultrasound protocol applied to hindlimb mouse muscle, the influence of gain settings on echo intensity (EI) and GSI index was analyzed.

Table 1 shows the results of the EI, GSI and TAI values. The WT and TIEG1 KO GSI varied from 0.62/0.65 to 0.36, respectively. These results provide referent GSI data as a function of the gain for

181 healthy (WT) muscle and dystrophic TIEG1 KO muscle (Fig. 3A-B). It can be noted that no
182 significant difference of GSI was measured between the two genotypes (Fig. 3C). This indicates
183 that the GSI parameter is not pertinent on US images to discriminate the two genotypes; another
184 imaging technique like MRI may be more suitable [8].

185 In WT mice, the results of the coefficient of variation (CoV) of EI as a function of the gain (G)
186 settings showed a higher mean (44.1) for the gain applied at 40% compared to the other ones (from
187 50% to 70%) (CoV between 11% and 20%). The same measurements were performed on the
188 TIEG1 KO muscle and a slightly higher range of CoV (between 14.1% to 52.8%) was found
189 compared to healthy (WT) muscle. This result demonstrated a less stable EI for dystrophic muscle.
190 It can be concluded that a gain between 50% and 70% could be used to accurately measure the EI of
191 healthy and pathological mouse muscle.

192 The TAI values remained stable as a function of the gain (Fig. 3 A-B) and were in the same
193 range (approximately 0.69) for both genotypes. This result reveals a main direction present within
194 the skeletal muscle which is a result of the fiber orientation. However, no significant TAI difference
195 was found between WT and TIEG1 KO muscle (Fig. 3 D). This result was previously confirmed
196 with MRI diffusion sequence (3 directions).

197

198 3.3 Functional properties of mouse hindlimb muscle

199 From the elastography images, no significant difference in mean stiffness or co-efficient of
200 variation was found. In particular, no significant difference in the stiffness was detected between
201 TIEG1 KO (36.1 ± 10.3 kPa) and WT (44.4 ± 13.4 kPa) muscle.

202 This is probably a limitation of the SWE technique applied to small mouse muscles, which analyzes
203 a group of muscles (gastrocnemius, peroneus brevis, soleus) having different physiological
204 behaviors, leading to a global elastic value while individual muscles could have been affected by
205 the lack of TIEG1 expression [11]. It can be noted that WT values are in the same range of values
206 found by Qin et al. (2014) for hindlimb mouse muscle using a MR elastography protocol.

207 **4. Conclusion**

208 This study put some light in construction and release of new experimental databases using a clinical
209 SWE ultrasound device for the characterization of the structural and functional properties of mouse
210 hindlimb muscle. The present study has provided an accurate ultrasound identification of the
211 different muscles located within the hindlimb. The major goal was to realize an exploratory analysis
212 of the collected data representing by: 1) the correct range of gain to apply for the ultrasound
213 measurement of muscle structure, 2) the structural and functional referent data for a group of
214 healthy mouse muscles, 3) the gray scale index, the texture anisotropy and the elasticity parameters.
215 Future studies using phantoms with parameters and geometry similar to mouse muscle could help
216 further the development and utilization of SWE technique for monitoring changes in skeletal
217 muscle in small animal models, over time and in response to treatment.

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221 **Ethical statement**

222 The protocol was approved by the French ministry of higher education, research and innovation
223 (Permit Number: DUO-4776) and the Mayo Clinic Institutional Animal Care and Use Committee
224 (Permit Number: A9615).

225

226 **Conflict of interests**

227 The authors declare no conflict of interests.

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229 **Acknowledgment**

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231 Government, through the program " Investments for the future" managed by the National Agency
232 for Research (Reference ANR-11-IDEX-0004-02)

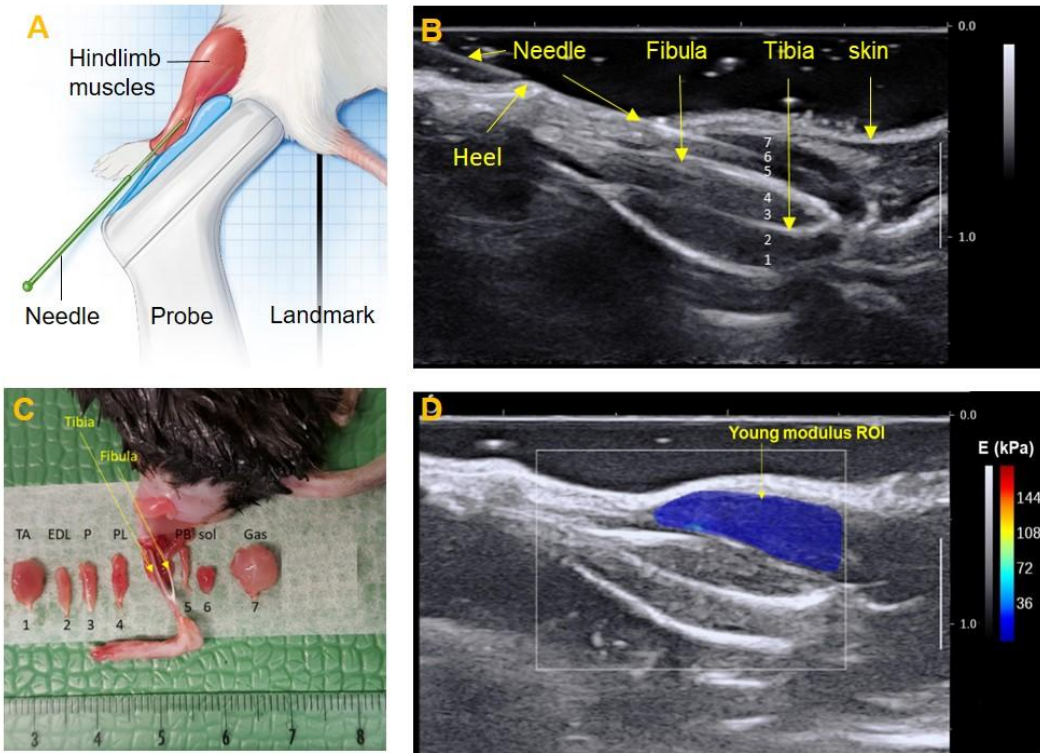
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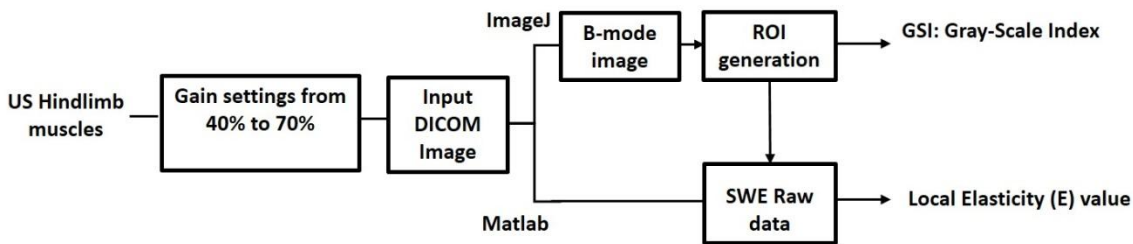
FIGURES:



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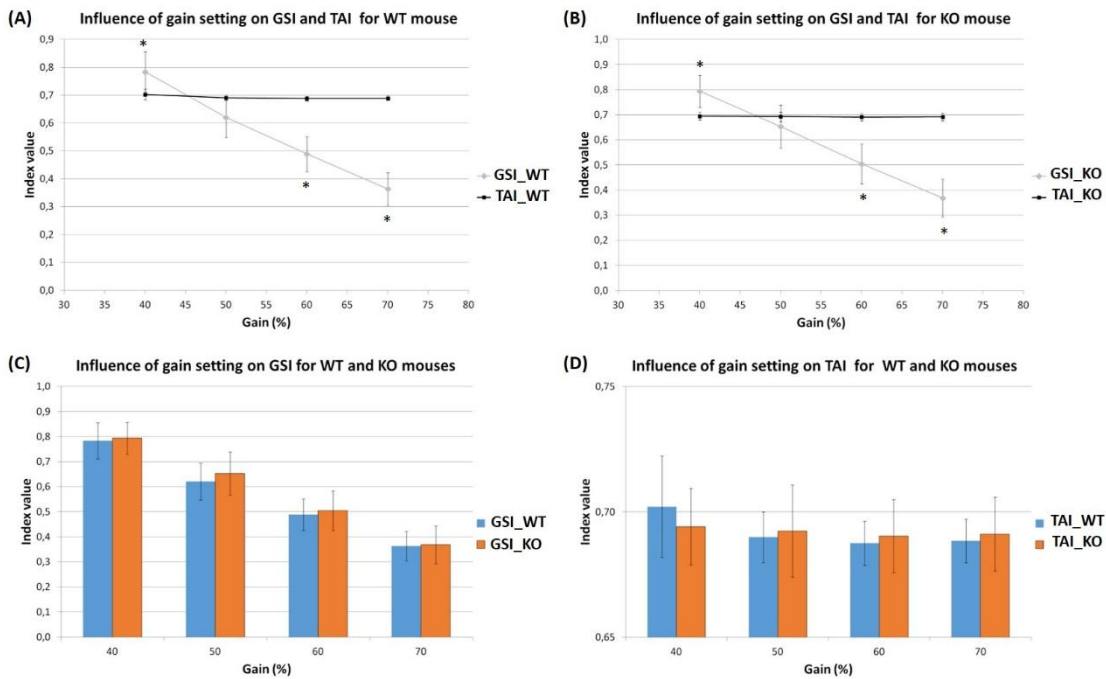
Fig. 1. (A) Ultrasound set up, (B) B-mode image of mice hindlimb with muscle identification using an acupuncture needle inserted in soleus. (C) Corresponding muscles (labeled from #1 to #7) identified on the B mode image. TA(#1): tibialis anterior, EDL(#2): extensor digitorum longus, P(#3): plantaris, PL(#4): peroneus longus, PB(#5): peroneus brevis, Sol(#6): soleus, Gas(#7): gastrocnemius. (D) Cartography of elasticity (E: Young modulus) with a ROI composed of 3 muscles (#5, #6, #7).

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Fig. 2. Flow chart of the proposed image processing. SWE: shear wave elastography. ROI: region of interest.



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290 **Fig. 3.** Influence of gain setting on gray-scale index (GSI) and texture anisotropy index (TAI) in
291 muscle of WT mouse (A) and KO mouse (B); Influence of gain setting on GSI (C) and on TAI (D)
292 for both type of mouse. *Significantly different from 50% gain. Vertical bars denote standard
293 deviation.

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295
296 **TABLES:**

297 Table 1. Means results of of muscle echo intensity EI, GSI and TAI for two types of muscle (WT
298 and TIEG1 KO).

299

Gain (%)	Mouse type		EI	CoV_EI (%)	GSI	TAI
40	WT (n=8)	Mean	55.3	44.1	0.783	0.702
		SD	18.4	10.0	0.072	0.020
	KO (n=8)	Mean	52.6	52.8	0.794	0.694
		SD	16.2	22.1	0.063	0.015
50	WT (n=8)	Mean	97.0	19.9	0.620	0.690
		SD	18.7	6.3	0.073	0.010
	KO (n=8)	Mean	88.5	27.0	0.653	0.692
		SD	21.8	13.7	0.085	0.018
60	WT (n=8)	Mean	130.4	15.6	0.489	0.688
		SD	15.9	3.7	0.063	0.009
	KO (n=8)	Mean	126.4	19.1	0.504	0.690
		SD	20.10	8.73	0.079	0.015
70	WT (n=8)	Mean	162.4	11.0	0.363	0.689
		SD	14.9	2.0	0.059	0.009
	KO (n=8)	Mean	161.1	14.1	0.368	0.691
		SD	19.2	2.9	0.075	0.015

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301 WT = wild-type; KO = knock-out; EI = echo intensity; TAI = texture anisotropy index; GSI = gray-
302 scale index; SD = standard deviation; CoV = coefficient of variation.

Figure 1

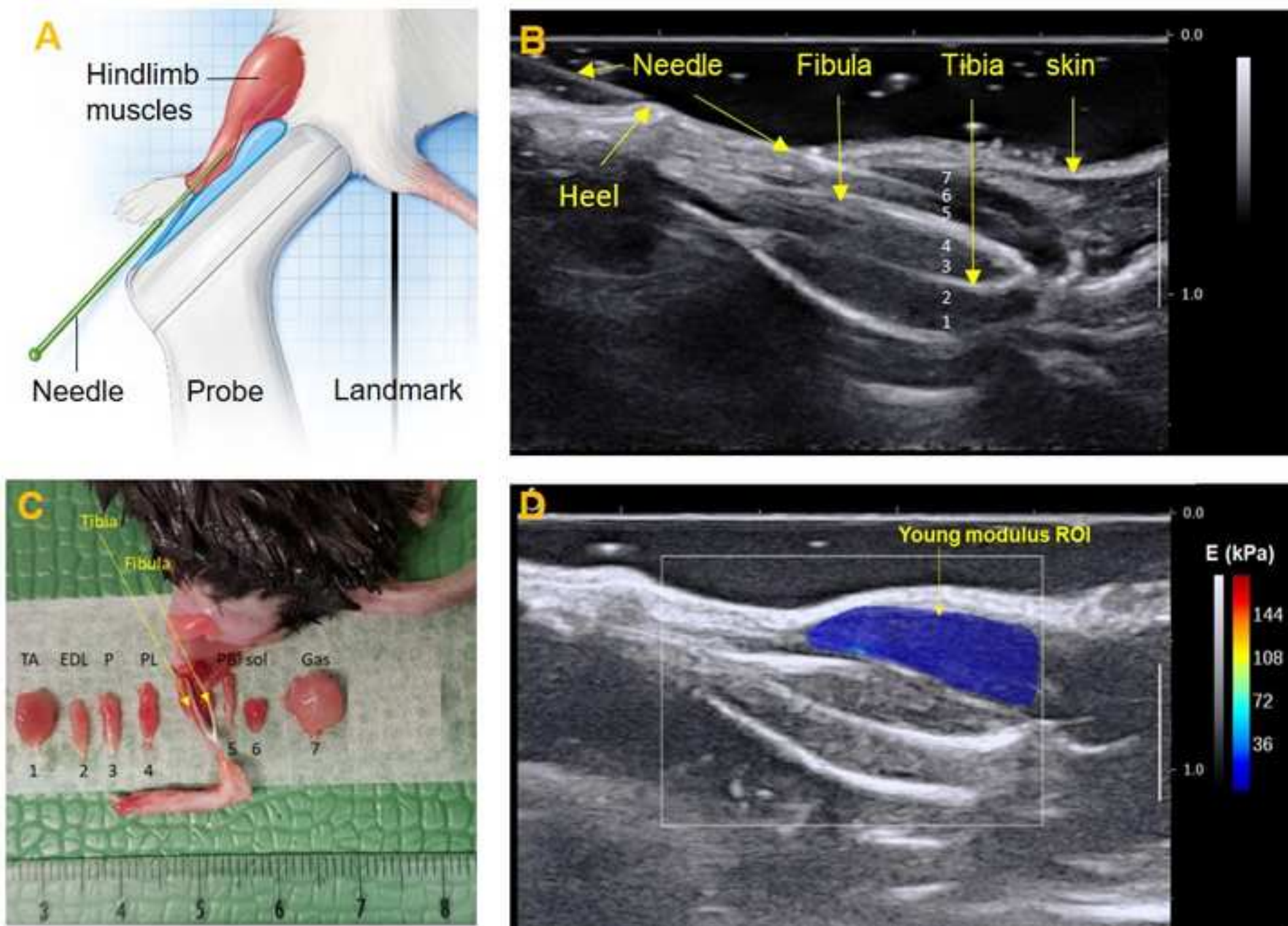


Figure2

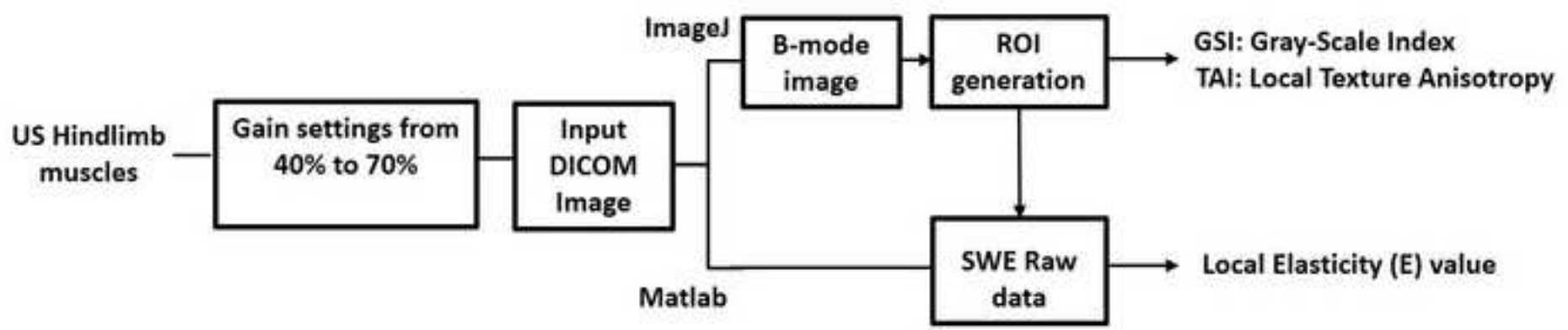
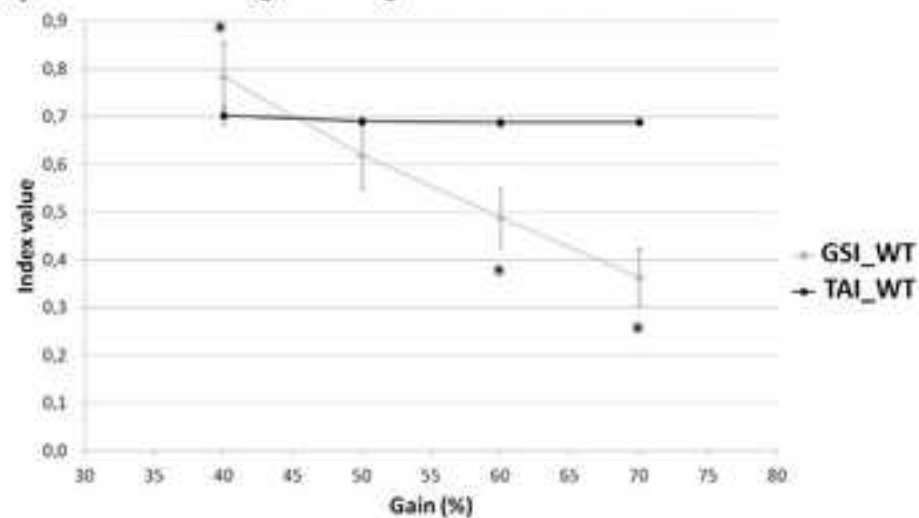
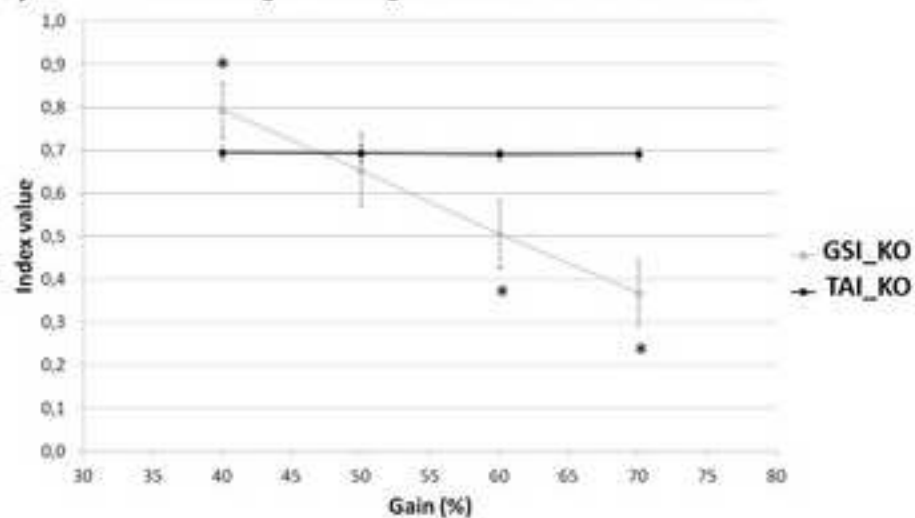


Figure3

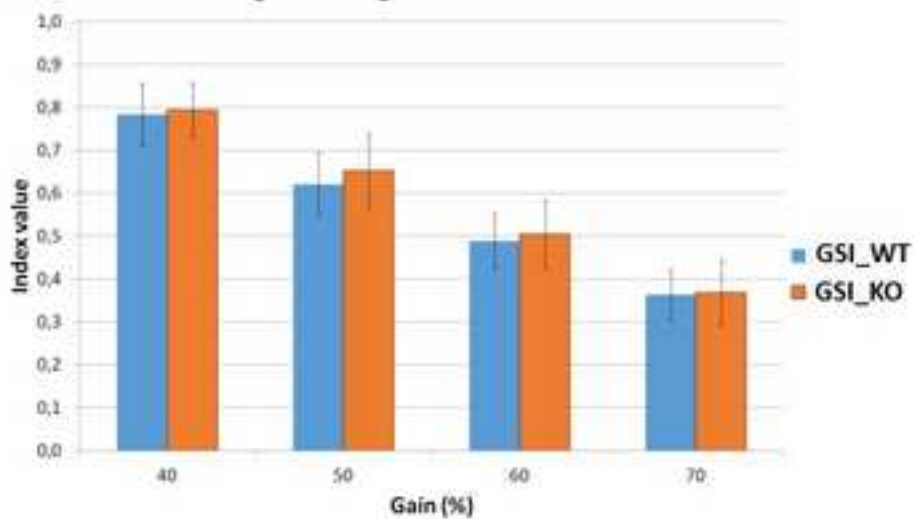
(A) Influence of gain setting on GSI and TAI for WT mouse



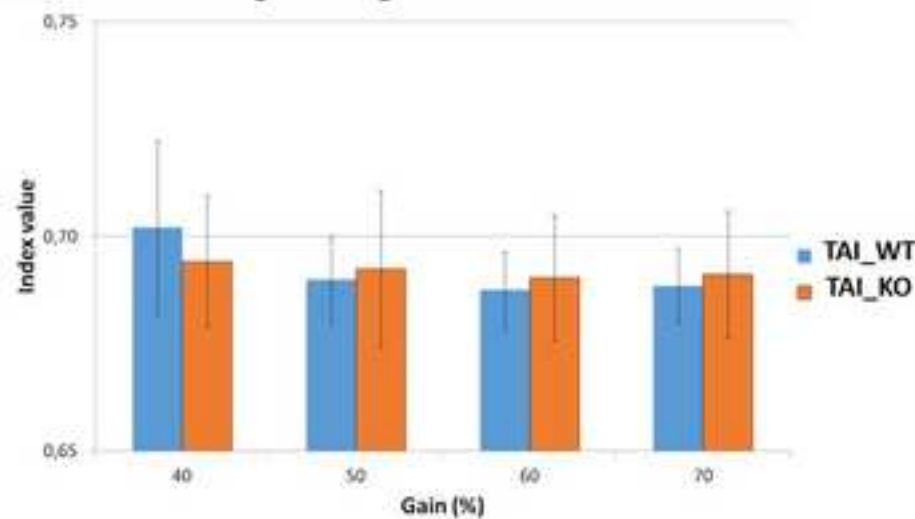
(B) Influence of gain setting on GSI and TAI for KO mouse



(C) Influence of gain setting on GSI for WT and KO mice



(D) Influence of gain setting on TAI for WT and KO mice



TABLES:

Table 1. Means results of of muscle echo intensity EI, GSI and TAI for two types of muscle (WT and TIEG1 KO).

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WT = wild-type; KO = knock-out; EI = echo intensity; TAI = texture anisotropy index; GSI = gray-scale index; SD = standard deviation; CoV = coefficient of variation.

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Conflict of interests

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Ethical statement

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