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Interdepartmental Research Center of Myology (cirMYO)
Centro Interdipartimentale di Ricerca di "MIOLOGIA" (cirMYO)
Biologia, Fisiopatologia, Clinica e Biotecnologie del Muscolo Scheletrico

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Subject: Proposals (RFP) on Clinical Criteria for the Diagnosis of Clinically Important Weakness Associated with Low Muscle Mass in Older Adults

Dear Maria Vassileva,

we are interested to be non-paid participants to the Project on "***Clinical Criteria for the Diagnosis of Clinically Important Weakness Associated with Low Muscle Mass in Older Adults*** ".

Despite our cohort of elderly is far to be "large", we are confident that *The Metabolic Disorders Steering Committee (MDSC) of the Biomarkers Consortium* will be interested in our new analytical tools to non-invasively monitor muscle structural/functional characteristics related to mobility weakness of elderly. Combining "*Muscle Color Three-Dimensional Tissue Segmentation & Composition"* and *"Functional Echomyography of Human Muscle"* it is possible to obtain bed-side non-invasive data on muscle tissue atrophy/degeneration/fibrosis that were previously collected only by serial muscle biopsies (something that ethical committees hardly grant).

As part of a larger European Task Force for Translational Myology, the Interdepartmental Research Center of Myology (University of Padova cirMYO, Italy) is coordinating the efforts of scientists/clinicians from the University of Padova, Italy (PI: Ugo Carraro, E-mail: ugo.carraro@unipd.it), the University of Chieti, Italy (PI: Giorgio Fanò, E-mail: fano@unich.it), the Ludwig Boltzmann Institute of Electrostimulation and Physical Rehabilitation, Department of Physical Medicine, Wilhelminenspital, Vienna, Austria (PI: Helmut Kern, E-mail: helmut.kern@wienkav.at), and the Department of Research and Development, HTS, Landspitali-University Hospital, Reykjavik, Iceland (Consultants: Thordur Helgason, E-mail: thordur@landspitali.is, and Paolo Gargiulo, E-mail: paologar@landspitali.is).

Subjects of the study in Chieti, Italy
The study was carried on 35 volunteers, healthy people (20 ♂ and 15 ♀) with a diagnosis of sarcopenia according to the Centers for Disease Control and Prevention (CDCP). The mean age of the 35 subjects is 70.9±4.7 years old.

Subjects of the study in Vienna, Austria
The study was carried on 23 volunteers, (12 ♂ and 11 ♀) either well-trained senior sportsmen (4) or with a diagnosis of sarcopenia according to the Centers for Disease Control and Prevention (CDCP). The mean age of the 23 subjects is 68.3±4,4 years old.

Methods. The inclusion criteria were as follows: diagnosis of sarcopenia, normal ECG and blood pressure, and absence of bone/joint or metabolic or cardiovascular diseases. Exclusion criteria included the presence of the above pathologies, essential hypertension, genetic or non-genetic muscular diseases, as well as diagnosis of respiratory and psychiatric diseases. None of the subjects were being treated with exogenous testosterone or other pharmacological interventions known to influence muscle mass. A new experimental session is in progress to repeat this experimental approach utilizing 20 new enrolled older subjects.

Training protocols
The subjects have been trained with 4 different training protocol:

1. Resistance training;
2. Endurance training;
3. Local vibration training;
4. Electrostimulation.

1- Resistance training

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3 male aged 70,7±3,1 years old and 3 female aged 72,0±3,5 years old have been trained for 12 weeks, 3 times a week. The resistance training protocol consisted in training on a Leg-extension and Leg-press machines. After a 10 min warm-up on a stationary bike, subjects performed:
1-4 weeks: 3 sets of 12 repetitions with a load of 60-70% of 1-RM;
5-9 weeks: 3 sets of 10 repetitions with a load of 75-80% of 1-RM;
9-12 weeks: 3 sets of 6-8 repetitions with a load of 80-85% of 1-RM.
2- Endurance training
3 male aged 70,7±2,1 years old and 5 female aged 70,8±4,3 years old have been trained for 12 weeks, 3 times a week. The endurance training protocol consisted in training on a stationary bike with training intensity given by target heart rate, monitored by a heart rate monitor.
Every training had a warm-up period of 5 min and a cool-down period of 5 min. After the warm-up at 60% of hart rate reserve (HRR), subjects trained at the following intensity given by heart rate:
1-4 weeks: 30' at 60-70% HRR; 5-8 weeks: 40' at 60-70% HRR; 9-12 weeks: 40' at 80% HRR.
3- Local vibration training
4 male aged 75,3±6,9 years old and 5 female aged 71,0±5,7 years old have been trained for 12 weeks, 1 time a week in the first 8 week and 3 times a weeks in the final 4 weeks.
The conditioning protocol consisted of local high-intensity vibration training. The VISS device (VISS, international patent by Bioelcom srl, Italy) comprised a tool capable of producing different frequency acoustic waves without affecting the set width. The tool is not an acoustic wave's generator, but a flux modulator. VISS has two components, specifically, a compressor with a 0 to 400 milliBar scale and a modulator that produces an undulating air flux to create acoustic waves through a two-way rotating valve. The transducer can develop a time-modulated sinusoidal (300 Hz). During the application of these force sequences, the patient did not have to maintain isometric contraction of the treated muscle. The experimental technique consisted of applying local mechano-sonorous vibratory stimulation on/over the skin overlying the distal part of the quadriceps close to the tendon insertion of *intermedius femoris*, *rectus femoris*, *vastus medialis* and *vastus lateralis* muscles. The duration of each application was 15 min at 300 Hz once a week from weeks 1 to 8, and three 15 min applications per week from weeks 9 to 12. We refer to this type of stimulation and protocol as 'local vibration training'.
4- Electrostimulation
10 male aged 70,0±4,9 years old and 2 female aged 65,5±0,7 years old have been trained for 8 weeks, 3 times a week. The electrostimulation training protocol consisted in 24 sessions of isometric bilateral neuro-muscular electrostimulation. The stimulation had a 18 min duration, consisting on 40 contractions of 6.25 s. The parameters of the electrostimulation were: Frequency: 75 hz pulse ; duration: 400 msec; intensity: variable; duty cycle:24%: 6.25 sec on; 20 sec off; single pulse: rise time 1.5 sec, steady time: 4 sec, fall time: 0.75 sec electrodes: anode: 10x5 cm, cathode: 5x5 cm electrodes positioning: vastus medialis (at the motor point) vastus lateralis (at the motor point).

Measurements
Two weeks before the training period, enrolled subjects were familiarized with the test session protocol. Isometric tests were performed a week before (pre-session) and a week after the conditioning period (post-session) to measure the bilateral maximal isometric strength of lower limbs, body mass index (BMI) and the circumferences of the thighs were taken with measuring tape at two sites , on the dominant leg of standing subject. The circumference sites were as follows: one-third of the subischial height up from the tibial–femoral joint space and the minimum circumference above the knee. Skin fold measurement was then taken at the anterior mid thigh using a skin fold caliper. Sixteen weeks after the end of the conditioning period of different training, the isometric test was repeated to monitor the lower limb strength level.
a- Isometric strength measurement
Isometric torque developed by knee extensor muscles was measured during maximal voluntary contractions using a Leg Extension machine (Panatta Sport, Apiro (MC) Italy) with a load cell (Globus Italia, Codognè (TV) Italy). Participants were seated with the trunk-thigh angle at 90° and knee joint angle of 90°. The subjects performed maximal voluntary isometric contraction of the knee extensors three times. Isometric contractions lasted 5 s each, and were separated by 2 min rest intervals. The highest value of torque (kg) reached was taken as isometric contraction strength. In each subject, variations of knee extension isometric force were expressed as a percentage of the pre-training value.
b- Cellular and molecular analysis of muscle biopsies
Muscle biopsies were obtained using a semi-automatic needle (Precisa 13 Gauge, Archis, Italy) from *vastus lateralis* at a level corresponding to one-third of the distance from the upper margin of the patella to the anterior superior iliac spine after local anesthesia with Lidocain 0.5% and without incision. Several samples were extracted from the same

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positioned insertion of the needle, placed in the suitable solutions, and employed for: (i) dissection and of single fibers for muscle mechanics; (ii) for gel electrophoresis and (iii) extraction of RNA for analysis of the transcriptional profile.

New Tools for Monitoring of Myopathies

Validated follow-up procedures of disease progression and regression/healing are basics for validation of diagnostic and treatment strategies. Experts recognize as "best-practice" what produce repeatable results, usually selecting the "easier approach". This strategy not produces necessarily clinical relevant results and the needed information. This need is a main justification for the increasing fragmentation of medical and non-medical knowledge in progressively focused sub-specialties, whose experts hardly continue to understand each others. Integration of the procedures and of the analytical approaches will better serve patients and will be a better investment of decreasing resources.

Beside standard clinical practice tuned to the different myopathies, MoM counts on two complementary analytical technologies tuned to the skeletal muscle tissue needs: 1. Muscle Three-Dimensional Tissue Segmentation & Composition Analysis [4-7] and 2. Functional Echomyography (Skeletal Muscle Ultrasonography) [14,16,19]. Additional methods could be discussed and integrated in the new MoM strategy.

Muscle volume and shape will be monitored by CT three-dimensional muscle segmentation and ultrasonography. Because of different physicochemical properties, the tissue composition can also be analyzed using CT and ultrasonographic approaches. By Functional Echomyography contraction dynamics of muscles are studied to answer what muscles or muscle parts are contracting under voluntary and/or electrical activation.

Main missing information in myology is the energy cost of muscle tissue healing. This may be approximated to the oxygen consumption of the muscle tissue at rest, comparing healthy and recovering muscle tissue. Would be blood flow to an anatomically defined muscle (or muscle part, if the terminal artery could be identified) be measurable using echo Doppler techniques? A mid-term LMN denervated muscle is, by definition, a permanent resting muscle, and thus will provide the experimental test to validate this non-invasive approach to determine the energy metabolism and thus the size of a an anatomical defined muscle.

We have preliminary evidence [14,16] that the perfusion dynamics of an intramuscular small-size (less than 0.5 mm diameter) artery identify long-lasting denervated muscle (or muscle part [14]), blood flow at rest would be an additional sound index of progression or recovery of muscle atrophy/degeneration.

In short, done in a resting muscle, the muscle blood flow would be correlated with its size and health. This remains an open issue and could be a main goal of the EU MoM Project to validate a reliable easy-to-be-performed measurement of the blood perfusion before, during and after inflammatory and non-inflammatory myopathies (muscle regeneration after traumatic or chemical injury, exercise-induced muscle soreness, polymyosites, fibromyalgia on one hand and atrophy-hypertrophy processes on the other).

In conclusion we would like to stress that: There are too many "old" controversial issues that need new approaches. We are confident that combining sound, but invasive "time zero" and "end-point" analyses (tissue biopsy [2,3,17,18] and three dimensional macro-morphometry [4-7]) with non-invasive, repeatable US analyses[14,16,19] some of this open questions will find a final answer: 1. Extent of denervation and spontaneous reinnervation. 2. Progression of atrophy to degeneration in denervated muscles. 3. Influence (positive/negative) of electrical stimulation on 1. (in well defined sub-groups of patients). 3. Translation to a wider population of healthy or diseased persons of the knowledge our interdisciplinary group of European Scientists and Clinicians accumulated during the EU Rise Project (Contract n. n.QLG5-CT-2001-02191) by treating the special case of irreversible complete muscle denervation and the ongoing Rise2-Italy Project, managed by cirMYO, that is extending the home-based FES training to cases of incomplete muscle denervation.

Muscle Color Three-Dimensional Tissue Segmentation & Composition

The terms medical imaging and image technology refers to techniques and processes employed to produce non invasively images of the internal aspect of the human body. In the clinical context, medical imaging is also known as clinical imaging and it is today one of the most valuable tool for diagnostic and clinical investigation. Most effort is made to improve associate technologies and techniques in specific field such as: instrumentation design, data acquisition, image processing and modeling. The three dimensional (3-Dimensional) visualization of the internal anatomy provides valuable information for the diagnosis and surgical treatment of much pathology. The main medical imaging technologies used in clinical practice allowing creation of three-dimensional images are: magnetic resonance imaging, computed tomography and medical ultrasound. In general, to produce 3-Dimensional images from these medical devices, many scans are made and the data processed by computers using special software. Finally the 3-Dimensional data can be further manipulated and processed to display specific anatomical region of interest. Here we present results of color 3-Dimensional imaging of anatomical defined skeletal muscles.

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Complementary analyses of physicochemical and functional properties of the skeletal muscles can be obtained, beside surface and needle electromyography, using magnetic resonance imaging and ultrasound approaches. For instance, using Functional Echomyography, stimulated muscles can be studied to establish their contraction properties and where and when they are contracting1. Blood flow characteristics of intramuscular vessels can also be analyzed. Using magnetic resonance imaging, the volume and shape of muscles and their tendons can be isolated and analyzed precisely.
In previous studies, the effects on muscular cross-sectional area of lower motor neuron denervation in spinal cord injury were monitored by means of computer tomography scans of the thigh, using the top of the trochanter major as the individual reference point. By scanning distally four further cross-sectional scans at increments of 10 cm, robust morphometry data of the patient's thigh were collected in the course of the European Union Project RISE, as described in [9,15]. This approach provides great clinical information, but its limitation is related to the fact that 5-10 scans over a volume of interest of 40-60 cm are not enough to build 3-Dimensional models of bone, muscle and muscle fascia, subcutaneous adipose tissue and other connective tissues.
In the present paper muscle tissue in normal and denervated subjects has been studied using spiral computer tomography scans and special medical imaging techniques. The target for the electrical stimulation is mainly the quadriceps femoris which includes four muscles on the front of the thigh: rectus femoris, vastus lateralis, vastus medialis and vastus intermedius. Rectus femoris occupies the middle of the thigh and it is certainly more exposed to the stimulation [3,13]. Beside it has a definite shape which can be isolated and studied very precisely. In our previous studies, segmentation techniques and special computational tools have been developed and used to isolate rectus femoris from other muscle bellies and to analyze muscle wasting and restoration in a novel way [5,8].
We describe a 3-Dimensional imaging approach using all the data gathered scanning with a spiral computer tomography the lower limbs of normal and spinal cord injury subjects in order to monitor muscle atrophy and degeneration due to long-term denervation and recovery of muscle structure as effect of a home based Functional Electrical Stimulation treatment. Particular attention is here dedicated to the cortical fibrous layer of the muscle (perimysium) that participates to both the long-term fibrous degeneration of the denervated muscle and to its recovery by Functional Electrical Stimulation.

Methods
Quantitative color 3-Dimensional computer tomography imaging obtained from data gathered scanning the patient's lower limbs with spiral computer tomography (see below) were performed at the Department of Research and Development, HTS, Landspitali-University Hospital, Reykjavik, Iceland. Clinical and functional assessments for the enrollment of the patients in the European Union project RISE, as well as follow-up and muscle biopsies, were performed at the Wilhelminenspital, Vienna, Austria. Light microscopy analyses were performed at the University of Padova, Italy.

Quantitative Color 3-Dimensional Computer Tomography Imaging
3-Dimensional data are gathered scanning the patient's lower limbs with spiral computer tomography. The scan starts above the head of the femur and continues down to the knee joint, both legs being covered by one scan. Slide increment is set to 0.625 mm resulting in a total of about 750–900 computer tomography slices, depending on the patient's size. Each slice consists of 512 × 512 pixels, and each pixel has a gray value in the Hounsfield Unit scale of 4096 gray-scale values, meaning that it is represented with a 12-bit value. A total data set from a single scan is therefore 512 ×512 ×750 ×12 = 2.36 Giga Bites. This data set allows a complete 3-Dimensional description of the tissue, including the muscles and bones in both upper legs. The size of the volumetric element (Voxel) in the data set is approximately 0,7 cubic millimeters, therefore the computer tomography number assigned to the voxel represents somehow an average of the different biological elements, which may be present within a single elementary volume. For instance, in the case of normal muscle tissue such voxel would contain the transverse section of 20 up to 50 muscle fibres, which is approximately one tenth of the volume analyzed with the typical needle muscle biopsy harvested according to Kern et al. 2004 [9], where a section of around 0,02 quadric millimeters presents the cross sections areas of about 50 fibers.
The results of this micro structural analysis are presented both as the percentage of different tissues (muscle, loose and fibrous connective tissue and fat) in the total volume of the rectus muscle and as 3-Dimensional muscle reconstructions displaying the first cortical layer of voxels that describe the muscle perimysium.
Special software tools were used (MIMICS software, www.materialise.com) and big efforts were paid to isolate and monitor rectus femoris in normal thighs. 3-Dimensional reconstruction is possible in rectus femoris even when it is severely degenerated due to long-term denervation: denervated musculature loses mass, volume and shape, becoming

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almost unrecognizable with respect to healthy muscles. Rectus femoris instead remains roughly recognizable during degeneration.
By using computer tomography data, biological tissues can be discriminated and analyzed. Specific attenuation values are assigned to individual volumetric element (voxel) depending from the relative transparency to the passage of X-rays through the volume element. The degree of attenuation depends on the energy spectrum of the x-rays as well as on the average atomic number of the mass density of the patient tissue. Most computers display hardware that requires integer numbers and therefore the linear attenuation coefficients are rescaled to an integer range that encompasses 4096 values, between -1000 and +3095. This scale is called computer tomography number or Hounsfield unit. Dense tissue, such as bone, has large positive computer tomography number while negative computer tomography numbers are typical for air spaces, lung tissues and fatty tissues. Muscles are normally displayed with computer tomography number between 50 and 100 Hounsfield units, though within a normal muscle belly there are also other tissue elements such as connective tissue and fat, which are coded with much lower computer tomography number. The different tissue elements express their specific computer tomography number if such tissue occupies completely the pixel area; otherwise the computer tomography number will be an average between different values. This fact explains the wide range of values present inside a data set and suggests the definition of various intervals to study muscle structural changes. Therefore, to monitor and estimate the tissue composition into the stimulated muscle volume, the computer tomography number present within the segmented volume are subdivided in four Hounsfield units intervals and displayed with different colors (Figure 1): [-200 – -10], [-9 – 20], [21 – 40] and [41 – 139] representing respectively fat (yellow), connective tissue (cyan), low dense/atrophic (pink) and normal muscle fibres (red).
The results from the segmentation, tissue discrimination and 3-Dimensional reconstruction are seen in Figures 1 and 2, where only the first cortical layer of voxel describing the muscle perimysium is depicted in the pictures. We would like to stress that these 3-Dimensional pictures show the tissue distribution at the level of the muscle surface (perimysium), which could possibly not coincide with the distribution on the whole muscle volume.

Functional Echomyography of Human Muscle (by Muscle Ultra Sonography)
Denervation of limbs occurs when there is a trauma to peripheral nerves and roots. The muscles lose mass rapidly, and much of the cross section becomes occupied by non contractile tissue, notably collagen and fat. The first goal of the Rise2-Italy Project is to identify among the clinical cases of the Physical Medicine and Rehabilitation Unit of the University of Padua a group of subjects with permanent injuries (complete or incomplete) of arm/leg skeletal muscles aimed at extending the results of the European Project RISE [2,9,11,12]. Demonstration that a high-power electrical stimulator associated with large surface electrodes induces single (twitch) or sustained (tetanus) contractions in long-term denervated human muscles of paraplegics, open the opportunity to obtain trophic effect and some functional recovery of denervated muscle in patients with severe muscle atrophy secondary to peripheral nerve lesions.

Materials and Methods
The eligible patients suffered traumatic injuries to plexus or single nerve (e.g., circonflessus or femoral nerve). At enlistment, shoulder or leg muscles of the patients do not respond to the clinical stimulation protocols for innervated muscle (twitch stimulation with 0.5 msec long impulse at 5-20 V/mAmp, or tetanising currents). Using an electrical stimulator for denervated muscle (i.e., with an adequate power) that discharges triangular waves 150-200 msec long at 20-80 mAmp to large surface wet electrodes (20x40cm, in Lap case) the denervated muscle produces repeatedly single contractions (twitch training). Patient will be revaluated monthly to verify if the denervated muscle partially recovers its excitability to a sufficiently short stimulations (duration <50msec), to be able to respond to short trains of impulse at 10-20 Hz frequency to perform tetanic contractions 2-3 sec long. This electrical stimulation protocol (5 times a week) will not replace, but complement the standard University of Padua Rehabilitation Unit protocol that includes passive and active functional rehabilitation. Extent of innervation/reinnervation will be checked with periodic EMG. To monitor changes in thickness and tissue composition of trained muscles ultrasound scan will be performed before and every three months during the 12 months of programmed treatment. In association the patient will do an echocolordoppler to observe the muscle perfusion and the change of the resistivity index (RI) before, during and after the stimulation. With this test is also possible to see the contraction of the muscle during the electrostimulation.

Altogether preliminary observations of this pilot study of the Rise2-Italy Project performed at the Padua Rehabilitation Unit suggests that Therapeutic Electrical Stimulation of the lower motor neuron denervated skeletal muscle could be extended from the rare Conus Cauda Syndrome to more common cases of peripheral nerve lesions (and very common old adults) with clinically significant results

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