



## Muscle Decline in Aging and Neuromuscular Disorders - Mechanisms and Countermeasures

Terme Euganee, Padova (Italy), April 13 - 16, 2016

Hotel Augustus, Viale Stazione 150 - 35136 Montebelluna Terme (Padova), Italy & Villa dei Vescovi, Luvigliano di Torreglia (Padova), Italy  
Phone +39 049 793 200 - Fax +39 049 793518 - [http://www.hotelaugustus.com/english/pages/hotel\\_augustus.php](http://www.hotelaugustus.com/english/pages/hotel_augustus.php) - E-mail: [info@hotelaugustus.com](mailto:info@hotelaugustus.com)  
Organizers: Ugo Carraro, Helmut Kern, Christiaan Leeuwenburgh, Werner Lindenthaler, Francesco Piccione, Carlo Reggiani, Marco Sandri

## ABSTRACTS

### WEDNESDAY April 13, 2016

Hotel Augustus, Viale Stazione 150, Montebelluna Terme (Padova), Italy

#### Exercise and FES in premature and late aging: Tutorial and get-together, H Kern, U Carraro, Chairs

#### Functional Electrical Stimulation (FES) of Aging Muscle

Helmut Kern

Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Department of Physical Medicine and Rehabilitation, Wilhelminenspital Wien, Austria Italy

E-mail: [wil.pys.kern-forschung@wienkav.at](mailto:wil.pys.kern-forschung@wienkav.at)

Physical exercise is known to have beneficial effects on muscle trophism and force production modulating signaling pathways involved in fiber type plasticity, muscle growth and mitochondria respiratory efficiency.<sup>1</sup> It has been shown that the decrease of muscle mass and strength observed in aging is linked to intracellular and extracellular abnormalities, that is, sarcoplasmic reticulum-to-mitochondria malfunctions and extracellular matrix metabolism, respectively.<sup>2</sup> Lifelong, high-level physical activity delays the medium and long term effects of aging.<sup>3</sup> Furthermore, when healthy seniors are exposed to regular neuromuscular Functional Electrical Stimulation (FES) training for a period of 9 weeks outcomes are an increase in muscle strength and muscle fiber size and, most importantly, an increase of fast fibers, the more powerful of skeletal muscle motor units.<sup>4</sup> Electron microscopy analysis of aging muscle show remodeling of mitochondrial apparatus as a consequence of fusion phenomena that are consistent with adaptation to physical exercise.<sup>5</sup> Altogether these results indicate that the FES-dependent beneficial effects on muscle force and mass are associated with changes in mitochondrial- and sarcoplasmic reticulum-related proteins involved in Ca<sup>2+</sup> homeostasis, providing new targets to develop therapeutic strategies to promote healthy aging.

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#### In-Bed Gym and FES: Fighting muscle weakness by take-home strategies

Ugo Carraro, Andrea Marcante, Alfons Baba, Francesco Piccione

IRCCS Fondazione San Camillo Hospital, Venice, Italy

E-mail: [ugo.carraro@ospedalesancamillo.net](mailto:ugo.carraro@ospedalesancamillo.net)

All permanent or progressive muscle contractility impairments (including age-related muscle power decline) need permanent managements. Beside eventual pharmacology therapy, a home-based physical exercise approach is helpful, in particular for bed-rested or bed-ridden patients.<sup>1</sup> Awaiting development of implantable devices for muscle stimulation, i.e., of electroceuticals as effective as pace-makers for cardiac arrhythmias or cochlear implants for hearing loss, education of hospitalized patients to take-home physical exercise managements is an effective low cost alternative.<sup>2</sup> Frail elderly due to advanced age or associated diseases are often hospitalized for long periods of time. There, their already modest amount of daily physical activity is reduced, contributing to limit their independence up to force them to the bed. Inspired by the proven capability to recover skeletal muscle contractility and strength by home-based Functional Electrical Stimulation (h-bFES) even in the worse cases of neuromuscular traumatic injuries,<sup>3-5</sup> but, mainly guided by common sense, we suggest a short (15-20 minutes) daily sequence of fifteen easy volitional physical exercises that are performed in bed (In-Bed Gym). If sedentary borderline persons challenge, but not stress, them-self, in a few days in hospital In-Bed Gym may increase muscle strength, fatigue resistance and independence in daily life activities. In surgical units this will grant standing of patients soon after operation, a mandatory measure to prevent thromboembolism risk. In-Bed Gym helps also to mitigate the bad mood that accompanies mobility limitations, strengthening patients' confidence in recovering partial or total independence.<sup>6</sup> Continued regularly, In-Bed Gym may help to maintain the independence of frail older people and to reduce the risk of the possible serious consequences of accidental falls. In-Bed Gym may also mitigate eventual arterial hypertension, a major risk factor in elderly people.<sup>7</sup>

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## THURSDAY April 14, 2016

Hotel Augustus, Viale Stazione 150, Montegrotto Terme (Padova), Italy

### Workshop "Circadian rhythms in skeletal muscle"

C Leeuwenburg, S Schiaffino, Chairs

Introduction by S Schiaffino, Padova, Italy

#### Circadian rhythms and the molecular clock in skeletal muscle

Karyn A. Esser

University of Florida, Gainesville, FL, US

E-mail: "Esser,Karyn" [kaesser@ufl.edu](mailto:kaesser@ufl.edu)

Disruption of circadian rhythms in humans and rodents has implicated a fundamental role for circadian rhythms in aging and the development of many chronic diseases including diabetes, cardiovascular disease, depression and cancer. The molecular clock mechanism underlies circadian rhythms and is defined by a transcription-translation feedback loop with Bmal1 encoding a core molecular clock transcription factor. Germline Bmal1 knockout (Bmal1 KO) mice have a shortened lifespan, show features of advanced aging and exhibit significant weakness with decreased maximum specific tension at the whole muscle and single fiber levels.<sup>1</sup> We tested the role of the molecular clock in adult skeletal muscle by generating mice that allow for the inducible skeletal muscle-specific deletion of Bmal1 (iMSBmal1).<sup>2</sup> Here we show that disruption of the molecular clock, specifically in adult skeletal muscle is associated with a muscle phenotype including reductions in specific tension, increased oxidative fiber type, and increased muscle fibrosis similar to that seen in the Bmal1 KO mouse. Remarkably, the phenotype observed in the iMSBmal1-/- mice was not limited to changes in muscle. Similar to the germline Bmal1 KO mice, we observed significant bone and cartilage changes throughout the body suggesting a role for the skeletal muscle molecular clock in both the skeletal muscle niche and the systemic milieu.<sup>3</sup> This emerging area of circadian rhythms and the molecular clock in skeletal muscle holds potential to provide significant insight into intrinsic mechanisms of the maintenance of muscle quality and function as well as identifies a novel crosstalk between skeletal muscle, cartilage and bone.

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#### Intrinsic and extrinsic control of circadian gene expression in skeletal muscle

Kenneth A. Dyar (1,6), Stefano Ciciliot (1), Guidantonio Malagoli Tagliazucchi (2), Giorgia Pallafacchina (1,4), Jana Tothova (1), Carla Argentini (1), Lisa Agatea (1), Reimar Abraham (1), Miika Ahdesmäki (5), Mattia Forcato (2), Silvio Biciato (2), Stefano Schiaffino (1,4), Bert Blaauw (1,3)

(1) Venetian Institute of Molecular Medicine (VIMM), Padova, Italy; (2) Center for Genome Research, Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; (3) Department of Biomedical Sciences, University of Padova, Italy; (4) Institute of Neurosciences, Consiglio Nazionale delle Ricerche (CNR), Padova; (5) Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Germany, (6) Present address: Molecular Endocrinology, Institute for Diabetes and Obesity, Helmholtz Zentrum München, Germany

E-mail: [kenneth.dyar@gmail.com](mailto:kenneth.dyar@gmail.com)

Physical activity and circadian rhythms are well-established determinants of human health and disease, but the relationship between muscle activity and the circadian regulation of muscle genes is a relatively new area of research. We compared the circadian transcriptomes of two mouse hind-limb muscles with vastly different circadian activity patterns, the continuously active slow soleus and the sporadically active fast tibialis anterior, in the presence or absence of a functional skeletal muscle clock (skeletal muscle-specific \*Bmal1\* KO). In addition, we compared the effect of denervation on muscle circadian gene expression. We found that different skeletal muscles exhibit major differences in their circadian transcriptomes, yet clock gene oscillations were essentially identical in fast and slow muscles. Furthermore, denervation caused relatively minor changes in circadian expression of most clock genes, yet major differences in expression level, phase and amplitude of many muscle circadian genes. Our studies suggest that a major physiological role of the skeletal muscle clock is to prepare the muscle for the transition from the light/inactive/fasting phase to the dark/active/feeding phase, in anticipation of periodic fluctuations in fuel supply and demand.

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2. Dyar KA, Ciciliot S, Tagliazucchi GM, et al. The calcineurin-NFAT pathway controls activity-dependent circadian gene expression in slow skeletal muscle. Mol Metab 2015;4:823-33. doi: 10.1016/j.molmet.2015.09.004. eCollection 2015.
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**Workshop “Diaphragm, structure and function”**

C Leeuwenburgh, C Reggiani, Chairs

**Bench to bedside research on critical illness myopathy (CIM) and ventilator induced diaphragm muscle dysfunction (VIDD): Underlying mechanisms and intervention strategies**

Lars Larsson

Department of Physiology and Pharmacology, Department of Clinical Neuroscience, Clinical Neurophysiology section, Karolinska Institutet, Stockholm, Sweden

E-mail: [lars.larsson@ki.se](mailto:lars.larsson@ki.se)

Muscle wasting in intensive care unit (ICU) patients may be related to the primary disease, but there is heterogeneity of underlying disease and pharmacological treatment among patients exhibiting similar outcomes. Thus, it is highly likely that a common component of ICU treatment per se is directly involved in the progressive impairment of muscle function and muscle wasting during long-term ICU treatment. The specific mechanisms underlying the muscle wasting and impaired muscle function associated with the ICU intervention are poorly understood in the clinical setting.<sup>1</sup> This is in part due to heterogeneity in pharmacological treatment, underlying disease, clinical history etc. There is, accordingly, compelling need for experimental animal models closely mimicking the ICU condition, including long-term exposure to mechanical ventilation and immobilization (lack of weight bearing and activation of contractile proteins, i.e., “mechanical silencing”). In this project, the muscle dysfunction, which by far exceeds the loss in muscle mass in limb and respiratory muscles in patients with CIM<sup>2,3</sup> and VIDD,<sup>4</sup> have been investigated in detail at the cellular and molecular levels in a rodent experimental ICU model, allowing detailed studies in an immobilized and a mechanically ventilated rat for long durations (up to weeks-months). The long-term scientific goals of the research are to: (a) define the causative agents, (b) develop sensitive, quick and accurate diagnostic tools and monitoring devices, and (c) develop efficient intervention strategies. This project, which focuses on the mechanisms underlying the severely impaired limb and respiratory muscle function (CIM and VIDD) in response to long-term mechanical ventilation and immobilization and the introduction of specific intervention strategies, constitutes a significant component of the attempt to achieve these long-term goals.

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**A finite element approach to diaphragm mechanics: multiscale modeling from fiber to whole muscle**

Lorenzo Marcucci (1,2), Piero Pavan (2,3), Luana Toniolo (1), Lina Cancellara (1), Arturo Natali (2,3), Carlo Reggiani (1,2)

(1) Department of Biomedical Sciences, University of Padova; (2) Center for Mechanics of Biological Materials, University of Padova; (3) Department of Industrial Engineering, University of Padova, Italy

E-mail: [carlo.reggiani@unipd.it](mailto:carlo.reggiani@unipd.it)

The diaphragm is the most important inspiratory muscle. It has a thin, dome-shaped structure, and separates the thoracic and abdominal cavities, mechanically interacting with the surrounding organs during its contractile function. Moreover, its muscle fibers have a complicated geometry, connecting to several ribs, lumbar vertebral bodies and to the central tendon. All these peculiar features make it a very challenging organ to be modeled and analyzed by means of finite element methods. We present our work aimed at creating a finite element model of the human diaphragm. A computational model based on a realistic, potentially patient specific, geometry, may help in the understanding of diaphragm related pathologies, such as chronic obstructive pulmonary disease (COPD), mechanical ventilation (MV) induced diaphragm inactivity, amyotrophic lateral sclerosis (ALS) or even for respiratory tumor motion tracking to reduce radiation treatment side effects. On the other side, several diaphragm pathologies are related to relevant modifications of the single fiber properties, such as contractile weakness, atrophy, or reduced cross sectional area. Moreover different fiber isoforms differently affect the whole muscle behavior. In the design of our model, we adopted a bottom-up approach, trying to explain the whole muscle behavior starting from the single fiber characterization. Therefore, we first developed a model for a single fiber based on a three elements Hill's model which is able to reproduce the classic protocols for the force velocity curve (isometric contraction followed by an isotonic contraction at different external forces) as well as the slack-test protocols (isometric contraction followed by a rapid shortening imposed to the fiber). We then characterized the parameters for both fast and slow human skeletal fibers, based on the original experimental data obtained in our lab, taking into account the effects of temperatures as single fiber mechanics is generally studied at low temperature (12-20°C). The characteristic width of the elements in the mesh of the diaphragm is several tens of microns, which means that each element likely include several slow and fast fibers. Then a characterization of the mixed fiber bundles is needed. We therefore characterized a mesh describing a bundle of fast and slow fibers to reproduce the mixing effect on the force velocity curve. The transversal connections linking each other adjacent fibers were taken into consideration. The mesh for the bundles was based on histological cross sections of diaphragm, which can be found in the literature [1]. Finally, we included our results in a 3-D mesh representing the human diaphragm. The bottom-up approach based on the real behavior of single bundles of fibers, is particularly useful to predict how different pathologies at the fiber level can influence the whole muscle performance.

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## Plasticity of mouse diaphragm: aging and response to endurance training

Silvia Quartesan (1), Lina Cancellara (1), Sara di Siena (2), Cinzia Calabrese (2), Fabio Naro (2) Carlo Reggiani (1)

(1) University of Padova, Department of Biomedical Science; (2) University of Roma "La Sapienza", Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Italy

E-mail: [carlo.reggiani@unipd.it](mailto:carlo.reggiani@unipd.it)

All skeletal muscles undergo an age-dependent loss of mass and functional deterioration. As follow-up of the functional impairment of lower limb muscles, the elderly experience limitations in locomotion and eventually lose the possibility of independent life. Accordingly, an impairment in respiratory function is the expected effect of the age-dependent decline of respiratory muscles. In spite of its essential role in breathing the age-dependent modifications of diaphragm muscle (DIAM), which is the main inspiratory muscle, have received less attention than limb muscles (Greising et al 2013, Elliott et al 2015). In this study we aimed to investigate the age-related changes of the mouse diaphragm and the possible impact of moderate endurance training. The respiratory function in mice is very different compared to humans. Breathing in mice as in other small mammals is characterized by very high frequency (400-500/min) and low tidal volume (<0.5 ml/breath). Thus, the murine diaphragm is a fast muscle, characterized by aerobic metabolic activity, which allows it to avoid fatigue. Murine diaphragm is particularly susceptible to damage when dystrophin or other proteins linked to dystrophin are absent (Stedman et al 1991, Randazzo et al 2013, Giacomello et al 2015). In spite of those significant differences, murine muscle can be considered a useful model to study human diaphragm. Three groups of healthy female CD1 mice were studied: young (Y, 4 months), old (O, 20 months), and oldest-old (OO, 32 months). In each group, two subgroups were formed: sedentary and trained. Training protocol included moderate intensity running on treadmill (13 cm/sec, for 30 minutes) 5 days/week for 8 weeks. After mice sacrifice, DIAM was dissected and immediately stored at -80°C. Proteins were analysed with 8% SDS-PAGE (Talmadge and Roy, 1993) to separate myosin heavy chain (MyHC) isoforms and with 10% SDS, followed by Western Blot and staining with antibody specific to PLIN5, TOM20 and Actin. RNA was prepared for Real Time PCR. The analysis of MyHC isoforms expression revealed a predominance (80-90%) of fast intermediate isoforms (2A and 2X) with minor components of slow/1 and fast 2B isoforms. Aging of sedentary mice was accompanied by an increase of slow MyHC (from 4% at 4 mo to 9% at 32 mo), a decrease in fast intermediate 2A and 2X (from 91% at 4 mo to 85% at 32 mo), a minor variation of fast 2B MyHC (from 4% to 6%). Training induced a very small fast-to-slow shift in 4 months mice and, surprisingly, an opposite, slow-to-fast transition in 32 mo old mice. The mitochondrial density evaluated by the TOM20/actin ratio was virtually unchanged in the sedentary mice of the 3 age groups. Training caused opposite effects in young and old mice: in the young the mitochondrial density increased two folds in trained mice compared to sedentary controls, whereas in the old mice, training caused a significant reduction in mitochondrial density. The presence of adipocytes in the muscles was evaluated using PLIN5 as a marker. The concentration of PLIN5 showed an increase with age (approximately 2 folds) in both sedentary and trained mice. In conclusion, our results show that DIAM undergoes an age-dependent fast-to-slow fibre type transition as most of muscles of the mouse. This is accompanied by a preserved mitochondrial density. Training can revert the age-dependent change in myosin

isoform expression but is not able to induce in the elderly the mitochondrial biogenesis which can be observed in the young

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## 7-day denervation-atrophy is also absent in emi-diaphragm of oldest rats

Elena Germinario (1), Barbara Ravara (1,2), Valerio Gobbo (3), Ugo Carraro (4), Daniela Danieli

(1) Interdepartmental Research Center of Myology (CIR-Myo), Department of Biomedical Sciences, University of Padova, Italy; (2) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Vienna, Austria; (3) Institute of Neuroscience, Consiglio Nazionale delle Ricerche, Padova (Italy); (4) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy

E-mail: [daniela.danieli@unipd.it](mailto:daniela.danieli@unipd.it)

Denervated left emi-diaphragm of adult rats is well known to paradoxically increase in weight during the first ten days after denervation, then it atrophy as any other denervated muscle.<sup>1</sup> Increased activity of eIF-2 initiation factor accounts at least in part for the enhancement of protein synthesis in 2-day denervated emi-diaphragm of adult rats.<sup>2</sup> Nothing is known about the behavior of the denervated emi-diaphragm in oldest (30-month) rats. We compared in two groups of four animals (3-month adults vs 30-month oldest male rats) the effect on functional and structural properties of 7-day denervation in the diaphragm and in denervated EDL and Soleus leg muscles. At 30-month the body weight of the rats is high significantly heavier than that of the 3-month animals (gr 289+/-44 vs 401+/-38, mean+/-SD, p< 0.001). Despite the increase in body weight the weight of the innervated EDL, and SOL muscles are lower in the oldest rats. Equal or slightly lower are also sizes of muscle fibers in 30-month innervated EDL, SOL and diaphragm. Contrastingly, the response to 7-day denervation is muscle related: EDL and SOL leg muscles show a 15% decrease in muscle fiber size, while the muscle fiber of denervated left emi-diaphragm are indistinguishable from contralateral innervated muscle in 3-month and 30-month rats. Of note is the fact, that in the 7-day denervated left emi-diaphragm of the oldest rats (30-month) slow-type muscle fiber present a 5.7% highly significantly increase in fiber size, and that in two out of the four rats total muscle fiber size analyzed in semi-thin section after fixation for electron microscopy present highly significant increase (+ 14.5 %) when compared with the contralateral innervated emi-diaphragm. On the other hand, muscle



fibers of 7-day denervated EDL and SOL of the same rats show the expected decrease in size (-18.7 %). In conclusion, both young and old diaphragms appear to be unresponsive to 7-day denervation atrophy.

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## Workshop "Neuromuscular adaptations to exercise and ES in aging"

Lee Sweeney, Marco Sandri, Chairs

## CIR-Myo - Lecture 1

### Autophagy and the control of neuromuscular junction

Marco Sandri, Padova, Italy

Francesca LoVerso (1), Silvia Carnio (1), Rüdiger Rudolf (5), Marco Sandri (1,2,3,4)

(1) Venetian Institute of Molecular Medicine, Padova, Italy; (2) Department of Biomedical Sciences, University of Padova, Italy; (3) Institute of Neuroscience, Consiglio Nazionale delle Ricerche, Padova, Italy. (4) Department of Medicine, McGill University, Montreal, Canada; (5) Institut für Toxikologie und Genetik, Karlsruhe Institute of Technology.

E-mail: [marco.sandri@unipd.it](mailto:marco.sandri@unipd.it)

Neuromuscular Junction (NMJ) is the synapse that connects motor-neuron with skeletal muscle and its stability is critical for muscle contraction. Inherited or acquired conditions that perturb the components of this unit lead to weakness, denervation and, ultimately, paralysis.<sup>1,2</sup> The signals that control such complex/critical structure in adulthood are largely unknown. Here we show that a retrograde signal is released from adult skeletal muscles to maintain a stable and functional NMJ. An efficient autophagy system is required for a functional NMJ because it controls MuSK clustering<sup>3</sup> Impairment of autophagy or inhibition of MuSK leads to NMJ dismantling and denervation. These results identify MuSK-autophagy axis as a retrograde signal that is required for NMJ maintenance and function in adulthood.

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## Autophagic flux of CHRN is regulated by RAB5-GTPase and T145 phosphorylation level of SH3GLB1 at mouse neuromuscular junction in vivo

Franziska Wild (1,2,3), Muzamil Majid Khan (1,2,3), Rüdiger Rudolf (1,2,3)

(1) Interdisciplinary Center for Neuroscience, University of Heidelberg, Germany; (2) Institute of Molecular and Cell Biology, Mannheim University of Applied Science, Germany; (3) Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Germany  
E-mail: [r.rudolf@hs-mannheim.de](mailto:r.rudolf@hs-mannheim.de)

Endolysosomal carriers containing nicotinic acetylcholine receptors (CHRN) are either recycled (1) or degraded via autophagy during atrophic conditions (2, 5). However, regulatory processes underlying their processing have remained elusive. We have recently shown that internalized CHRN are accompanied by TRIM63 (MuRF1) and SH3GLB1 (3, 4). Here we further dissected the role of SH3GLB1 in regulating the autophagic flux of CHRN. Upon sciatic denervation, we found a tight regulation of SH3GLB1/phosphorylated-T145 (p-T145) SH3GLB1 protein amounts. While p-T145 SH3GLB1 levels remained constant upon induction of muscle atrophy, the non-phosphorylated SH3GLB1 protein was increased. Overexpression of a T145 phosphomimetic mutant (T145E) of SH3GLB1 slowed down the processing of CHRN endolysosomes while as T145 phosphodeficient mutant (T145A) of SH3GLB1 strongly augmented it. The slow processing of CHRN endolysosomes brought about by T145E was rescued upon co-expression of the early endosomal orchestrator RAB5, suggesting a role of SH3GLB1 in regulating CHRN endocytic trafficking at steps upstream of autophagosome formation.

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## CK2-dependent phosphorylation in skeletal muscle regulates neuromuscular junction stability and mitochondrial homeostasis

Said Hashemolhosseini

Institut für Biochemie, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany

E-mail: [said.hashemolhosseini@fau.de](mailto:said.hashemolhosseini@fau.de)

Protein kinase CK2, a pleiotropic serine/threonine kinase, plays an important role in many different biological processes inside of cells.<sup>1</sup> Conditional muscle-specific CK2 mutant mice lack grip strength and show muscle fatigability.<sup>2</sup> We identified the role of CK2 in skeletal muscle cells as a regulator of neuromuscular junction maintenance



by phosphorylation of different protein members of the postsynaptic apparatus.<sup>2,3</sup> Moreover, CK2 is involved in ensuring proper mitochondrial homeostasis in skeletal muscle fibers by fine-tuning mitochondrial protein import through the translocase of the mitochondrial outer membrane. In absence of CK2-dependent phosphorylation of a mitochondrial outer membrane translocase protein, muscle fibers undergo accelerated mitophagy, as demonstrated by an up-regulated PINK/Parkin/p62 pathway.

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### Spatial distribution of neuromuscular junction components. Pretzels are more complex than we have anticipated

Tomasz Prószyński

Laboratory of Synaptogenesis, Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland

E-mail: [t.proszynski@nencki.gov.pl](mailto:t.proszynski@nencki.gov.pl)

Mammalian neuromuscular junctions (NMJs) undergo a postnatal topological transformation from a simple oval plaque to a complex branch-shaped structure often called a “pretzel”. Although abnormalities in NMJ maturation and/or maintenance are frequently observed in neuromuscular disorders, such as congenital myasthenic syndromes (CMSs), the mechanisms that govern synaptic developmental remodeling are poorly understood. It was reported that myotubes, when cultured aneurally on laminin-coated surfaces, form complex postsynaptic machinery, which resembles that at the NMJ. Interestingly, these assemblies of postsynaptic machinery undergo similar stages in developmental remodeling from “plaques” to “pretzels” as those formed in vivo. We have recently demonstrated that podosomes, actin-rich adhesive organelles, promote the remodeling process in cultured myotubes and showed a key role of one podosome component, Amotl2. We now provide evidence that several other known podosome-associated proteins are present at the NMJ in vivo and are located to the sites of synaptic remodeling. Additionally, we identified proteins that interact with Amotl2 in muscle cells. We show that two of them: Rassf8 and Homer1, together with other podosome components, are concentrated at postsynaptic areas of NMJs in the indentations between the AChR-rich branches. Our results provide further support for the hypothesis that podosome-like organelles are involved in synapse remodeling and that Rassf8 and Homer1 may regulate this process.

This research was supported by grants 2012/05/E/NZ3/00487, 2013/09/B/NZ3/03524 and 2014/13/B/NZ3/00909 from the National Science Centre (NCN).

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### Mitochondrial Alarmins are key intercellular signals in the degeneration & regeneration of the neuromuscular junction

Michela Rigoni (1), Elisa Duregotti (1), Samuele Negro (1), Michele Scorzeto (1), Irene Zornetta (1), Bryan C. Dickinson (2), Christopher J. Chang (2), Cesare Montecucco (1)

(1) Department of Biomedical Sciences, University of Padova, Italy;  
(2) Department of Chemistry and Molecular and Cell Biology, University of California, Berkeley, USA

E-mail: [cesare.montecucco@gmail.com](mailto:cesare.montecucco@gmail.com)

The neuromuscular junction is one of the few human tissues capable of complete regeneration after major damages. We have set up a reliable model of acute degeneration of the motor axon terminals followed by complete recovery of function.<sup>2</sup> We have found that alarmins released by mitochondria of the degenerating nerve terminal are key factor that act on the perisynaptic cells and muscle fibre.<sup>2</sup> These cells are activated and release signals that act retrogradely on the nerve terminal inducing its regeneration. Some of these signals are currently being investigated by imaging and transcriptomics methods.

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### Introduction by Gerta Vrbova, London, Uk

#### Comparison of electrical stimulation to exercise,

Gerta Vrbova, Dept of Life Sciences UCL, London, UK

E-mail: [g.vrbova@ucl.ac.uk](mailto:g.vrbova@ucl.ac.uk)

Two fundamental differences exist between voluntary muscle contractions and those induced by electrical stimulation. During voluntary movements motor units are activated asynchronously and a strict hierarchical order of recruitment is always maintained where the smallest motor units are activated first followed by contractions of larger units. Thus during voluntary movement the largest motor units are least active and are used only during maximal effort. This order of recruitment is cancelled when electrical stimulation of the muscle is used; indeed due to the biophysical properties of the axons that innervate the muscle the largest motor units are activated preferentially and therefore the parts of the muscle that are usually used rarely are active most frequently. Thus during electrical stimulation it is the motor units that are normally least active that experience the biggest increase in their use and consequently the biggest change in their characteristic properties. Thus electrical stimulation by bypassing the hierarchical order of recruitment, indeed by reversing it, is able to activate those motor units and muscle fibres that can only be activated during most strenuous





exercise. It can therefore exploit the adaptive potential of muscle more efficiently than exercise and maintain much higher levels of activity over time than exercise. This enhanced activity is restricted to specific target muscles and is unlikely to have unwanted systemic effects. Finally, high amounts of activity can be imposed on a muscle from the beginning, since the CNS, cardiovascular and other systems will not interfere or limit the amount of activity carried out by the muscle, as is the case during exercise (see for review Pette and Vrbová, 1999).<sup>1</sup> On the other hand, there are several functions that electrical stimulation of individual muscle groups cannot accomplish and that are unique to exercise induced activity. During exercise-induced activity coordinated movement is carried out and it is therefore likely that the individual's skills in carrying out movement of this kind will improve. Thus, while exercise can improve coordination, electrical stimulation is unlikely to do so. In addition the flexibility of joints and lengthening of muscles can be improved by exercise but not by electrical stimulation. Particular exercise regimes such as Pilates and yoga are particularly effective in achieving these goals. Improvement of the cardiovascular system is also easier achieved by exercise. Nevertheless, it can be argued that having muscles that are less fatiguable than usual, an advantage that is readily achieved by electrical stimulation, enables the individual to exercise more efficiently and achieve all the goals regarding fitness more readily and in a shorter time.

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## CIR-Myo - Lecture 2

### Difference in muscular adaptation due to regular exercise or through electrical stimulation

Christiaan Leeuwenburgh

University of Florida, Gainesville, FL, US

E-mail: "Leeuwenburgh, Christiaan" [cleeuwen@ufl.edu](mailto:cleeuwen@ufl.edu)

Regular exercise and neuromuscular electrical stimulation (NMES) have been used in a variety of settings for different populations. Various modes of exercise (eccentric, concentric, resistance and aerobic) and NMES training regimes (localization, intensity, duration, frequency) exist for healthy subjects and athletes, patients in a variety of rehabilitation and preventive settings, either partially or in totally immobilized subjects. Both standard exercise interventions and NMES have been shown to be effective in preventing the decrease in muscle strength, muscle mass and the loss in oxidative capacity of skeletal muscles following multiple types of surgical (orthopedic) procedures. However, it is still not entirely clear whether biological adaptations are similar and the duration differences in their adaptation duration. We will discuss potential biological differences in adaptation at the neuromuscular junction and potential differences in bioenergetics adaptation. Future research needs to determine potential molecular differences and beneficial post adaptation differences.

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## FRIDAY April 15, 2016



Villa dei Vescovi, Luvigliano (Padova), Italy

Introduction by Carlo Reggiani, Padua, Italy

## CIR-Myo – Lecture 3

### Therapeutic Targets for Muscular Dystrophy

Lee Sweeney, University of Florida, Gainesville, US

H. Lee Sweeney, Margaret M. Sleeper, Sean C. Forbes, Ai Shima, Glenn A. Walter, and David W. Hammers

Myology Institute, University of Florida, Gainesville, FL, US

E-mail: "Lee Sweeney" [lsweeney@mail.med.upenn.edu](mailto:lsweeney@mail.med.upenn.edu)

Duchenne muscular dystrophy (DMD) is caused by loss of the force transmitting and membrane complex organizing protein, dystrophin, and is characterized by progressive muscle deterioration with failed regeneration and replacement with a fatty-fibrous matrix.<sup>1</sup> Dystrophin replacement therapies to date have shown only limited ability to slow the disease process, and thus therapeutics targeting other aspects of the disease, which can be used in combination, are needed. One potential target is nuclear factor  $\kappa$ B (NF $\kappa$ B), which is upregulated in the DMD muscles.<sup>2</sup> We examined a novel class of NF- $\kappa$ B inhibitors in mdx mouse and golden retriever muscular dystrophy (GRMD) dog models of DMD. These orally bioavailable compounds improved the phenotype of voluntarily run mdx mice, in terms of amount of activity, muscle mass and function, inflammation, and fibrosis. Surprisingly, the muscles were also more resistant to contraction-induced damage, which we demonstrated was significant increases in dysferlin, a protein required for membrane damage repair. We also evaluated the cardiac impact of a phosphodiesterase 5 (PDE5) inhibitor, tadalafil, which has been shown to improve blood flow in exercising skeletal muscles in mdx mice and human DMD patients.<sup>3</sup> Cardiomyopathy is a leading cause of mortality among DMD patients and is well modeled by the golden retriever muscular dystrophy (GRMD) dog model of DMD. Prophylactic use of the PDE5 inhibitor, tadalafil, improved GRMD histopathological features of the hearts, decreased levels of the pathogenic cation channel TRPC6, increased phosphorylation of TRPC6, decreased m-calpain levels and indicators of calpain target proteolysis, and elevated levels of the dystrophin ortholog, utrophin. The progressive loss of cardiac function was significantly slowed by these effects. These data demonstrate that prophylactic use of tadalafil delays the onset of dystrophic cardiomyopathy, which is likely attributed to modulation of TRPC6 levels and permeability and inhibition of protease content and activity, which results in higher levels of the protective protein, utrophin. Thus PDE5 inhibition and NF- $\kappa$ B inhibition are potential therapeutics to consider in developing a combinatorial approach to the treatment of DMD.



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10.00 **Mobility impairments in aging and myopathies, I**  
*Christiaan Leeuwenburg, Ugo Carraro Chairs*

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**Handling growth and glucose through muscle IGF-I**

**Elisabeth Barton**

University of Florida, Gainesville, FL, US

E-mail: "Barton, Elisabeth R" [erbarton@ufl.edu](mailto:erbarton@ufl.edu)

IGF-I and insulin are intrinsically connected through their actions on the IGF-I and insulin receptors to regulate blood glucose (1). Reduced circulating IGF-I can be compensated by heightened insulin, but chronically elevated insulin can lead to insulin resistance and ultimately diabetes. Further, increased circulating or local muscle IGF-I may enhance glucose uptake. If IGF-I from muscle and liver is equivalent, then loss of muscle IGF-I should result in a similar pathologic diabetic state (2). By extension, if muscle IGF-I is elevated, it may serve a protective role in glucose homeostasis, either through increased muscle mass providing a greater glucose sink (3), or through increased hybrid receptor activation by IGF-I (4). To address the impact that these factors have on metabolism, we elevated IGF-I by local AAV-IGF-I injections into both hindlimbs of adult male mice (5), and reduced muscle IGF-I through an inducible muscle specific deletion of Igf1. Mice were subjected to tests for body composition, glucose uptake, and energy expenditure compared to age-matched controls. It was not surprising that the hindlimb injections boosting IGF-I levels only in a small group of muscles did not alter the whole animal body composition. Further, when mice were subjected to treadmill running for 60 minutes, there were no significant changes in blood levels of glucose or lactate pre- or post-exercise. While increased muscle mass did not appear to alter basal glucose uptake, increased IGF-I content altered contraction induced glucose uptake in muscle when normalized to mass. To understand the consequences of diminished muscle IGF-I production, we generated mice with inducible muscle specific IGF-I deletion, with induction in adult mice. In mice with muscle specific deletion of Igf1, glucose levels following uphill treadmill running increased by 10%, in contrast to controls where blood glucose decreased by ~40%, supporting that glucose clearance is mediated in part through muscle IGF-I. Further, a marked impairment of glucose clearance occurred following a simple glucose tolerance test. Based on these results, we assert that the IGF-I produced by the muscle has an endocrine function, and like IGF-I produced by the liver, modulation of muscle levels of IGF-I will lead to changes in glucose homeostasis.

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**Therapeutic approach through liposomes carrying antioxidant in skeletal muscle**

**Rosa Mancinelli (1), Simone Guarnieri (1), Alessio Rotini (1), Viviana Moresi (2), Cecilia Bombelli (3), Simona Sennato (4), Stefania Fulle (1)**

(1) Dept. of Neuroscience, Imaging and Clinical Sciences, "G. d'Annunzio" University, Chieti-Pescara; (2) Dept. of Anatomy, Histology, Forensic Medicine and Orthopedics, "La Sapienza" University, Rome; (3) Dept. of Chemistry, "La Sapienza" University, Rome; (4) Dept. of Physics, "La Sapienza" University, Rome, Italy

E-mail: "Rosa Mancinelli" [r.mancinelli@unich.it](mailto:r.mancinelli@unich.it)

Sarcopenia is the age-related loss of muscle mass, strength and function leading to loss of muscle power, which in the end results in frailty and disability. At molecular level, sarcopenia is a complex condition characterized by insufficient antioxidant defense mechanism, increased oxidative stress and altered function of respiratory chain (Fulle S., 2005). It has been hypothesized that the accumulation of oxidative stress is also related to an impaired regeneration cooperating to the atrophic state that characterizes muscle ageing (Beccafico S., 2007). To the purpose, we investigated the myogenic process in satellite cells, the skeletal muscle stem cells, as myoblasts and myotubes collected by human Vastus Lateralis skeletal muscle of young and old subjects through needle-biopsies (Pietrangolo T., 2011). NBT and H2DCF-DA assays were used to measure O2•- and ROS production, respectively. Data revealed that oxidant species are more concentrated in elderly myoblasts compared to young ones. To evaluate if mitochondria are affected by ROS using JC-1 assay we found that in elderly myoblasts mitochondrial transmembrane potential decreases much more than in young ones probably due to their lower endogenous antioxidant abilities. Furthermore, MitoSOX™ Red reagent was used to measure directly O2•- in mitochondria. We found that in elderly myoblasts O2•- production is increased respect to young ones and the result is worsened in myotubes. Gene expression analysis revealed that genes involved in atrophic and ubiquitin-proteasome pathways were upregulation together with the dysregulation of the proliferative one suggesting an alteration at gene expression level in elderly myoblasts vs young ones (Pietrangolo T., 2009). In an attempt to ameliorate muscle regeneration in elderly mitochondrion-specific liposomes carrying antioxidant were synthesized. The toxicity of liposomes were tested on human satellite cells and C2C12 cells, a murine skeletal muscle cell line. Preliminary results demonstrated that liposomes made using DPPC 97.5%/BOLA 2.5% gave the lowest toxicity at 24-48-72 hours. Overall, if we need more data and further analysis, up to day our data suggest that oxidative stress impairs muscle regeneration in elderly subjects.





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### Molecular determinants of proteins localization to triadic junction

Enea Liguori, Daniela Rossi, Vincenzo Sorrentino

(1) Molecular Medicine Section, Department of Molecular and Developmental Medicine, University of Siena, Italy

E-mail: [vincenzo.sorrentino@unisi.it](mailto:vincenzo.sorrentino@unisi.it)

The sarcoplasmic reticulum (SR) is organized in longitudinal and junctional SR (j-SR). In skeletal muscle, this latter domain together with the T-tubules form specific junctions called triads, where proteins regulating the excitation-contraction coupling mechanism assemble. Junctophilins (JPs) are directly involved in the formation and maintenance of triads. Basically, they are anchored to the SR via their C-terminal transmembrane domain (TMD), while their N-terminus contains eight MORN motifs, which associate with the phospholipids of the T-tubules. Nevertheless, how JPs are targeted to triads is not known. The roles of the N-terminal and the C-terminal regions of JP1 in this process were investigated. Expression in primary myotubes and/or muscle fibers of JP1 deletion mutants lacking the TMD resulted in protein distribution at both the surface sarcolemma and the T-tubules, confirming that MORN motifs are involved in JP1 interaction with the sarcolemma, but are not sufficient to restrict its localization at the T-tubules. On the other hand, progressive deletion of the eight MORN motifs or even of the entire cytosolic region, did not affect JP1 localization at triads, indicating that the presence of the TMD is sufficient for JP1 localization at the j-SR. FRAP analysis performed on a GFP-TMD fusion protein expressed in myotubes indicated that this protein has a high mobility, suggesting the absence of strong protein-protein interactions occurring at the j-SR. Further work is needed to better understand the molecular mechanisms driving TMD-mediated JP1 localization at triads.

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### CASQ1 mutations in human skeletal muscle diseases

Valeria del Re, Valentina Poverino, Alessandra Gamberucci, Virginia Barone, Vincenzo Sorrentino

Molecular Medicine Section, Department of Molecular and Developmental Medicine, University of Siena, Italy

E-mail: [vincenzo.sorrentino@unisi.it](mailto:vincenzo.sorrentino@unisi.it)

Physical Calsequestrin (CASQ) is the major protein of the sarcoplasmic reticulum of striated muscle that binds Ca<sup>2+</sup> with high capacity and moderate affinity. CASQ exist as a monomer and polymers, depending on Ca<sup>2+</sup> concentration. CASQ switches from an unfolded state to a folded monomer when the ionic strength increases allowing the formation of front-to-front first and then back-to-back interactions in higher Ca<sup>2+</sup> concentrations. Recently we reported one mutation in the skeletal CASQ1 gene in a group of patients with a vacuolar myopathy characterized by the presence of inclusions containing CASQ1 and other SR proteins. The CASQ1 mutation (CASQ1D244G) affects one of the high-affinity Ca<sup>2+</sup>-binding sites of the protein and alters the kinetics of Ca<sup>2+</sup> release in muscle fibers from patients. Expression of the CASQ1D244G in myotubes and in mouse fibers causes the appearance of SR vacuoles containing aggregates of the mutant CASQ1 protein that resemble those observed in patients. Studies of Ca<sup>2+</sup> release showed an increase in Ca<sup>2+</sup> storage in CASQ1WT COS-7 transfected cells whereas no increase was observed in CASQ1D244G. Moreover both CASQ1WT and CASQ1D244G were expressed in bacteria, purified and analysed for their ability to polymerize at increasing Ca<sup>2+</sup> concentrations. The results obtained indicate that the CASQ1D244G protein polymerizes at lower Ca<sup>2+</sup> levels and more rapidly than CASQ1WT. These results suggest that the CASQ1D244G mutation interferes with the correct process of Ca<sup>2+</sup>-dependent protein polymerization causing altered intracellular calcium storage and the formation of protein aggregates.

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### Calcium Entry Units (CRUs): discovery of intracellular junctions that promote assembly of STIM1 and Orai1 in skeletal muscle

Simona Boncompagni

CeSI - Center for Research on Ageing & DNICS - Dept. of Neuroscience, Imaging, and Clinical Sciences, Univ. G. d'Annunzio of Chieti, Italy & Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Wien, Austria

E-mail: [s.boncompagni@phobos.unich.it](mailto:s.boncompagni@phobos.unich.it)

Store-operated Ca<sup>2+</sup> entry (SOCE), also referred to as capacitative Ca<sup>2+</sup> entry, plays an important role in intracellular Ca<sup>2+</sup> regulation. SOCE is a ubiquitous Ca<sup>2+</sup> entry mechanism, first described in non-excitable cells, that is triggered by depletion of intracellular Ca<sup>2+</sup> stores (endoplasmic/sarcoplasmic reticulum, respectively ER and SR). SOCE is coordinated by the interaction of stromal interaction molecule 1 (STIM1), which acts as the Ca<sup>2+</sup> sensor in the ER lumen,<sup>1</sup>



and Orai1, the  $\text{Ca}^{2+}$ -permeable channel in the plasma membrane (PM).<sup>2</sup> Specific Gap of Knowledge. SOCE is also well-documented in skeletal muscle,<sup>3</sup> where it limits muscle fatigue during repetitive fatiguing stimulation.<sup>4</sup> Several studies suggest that STIM1-Orai1 coupling occurs within the pre-formed SR-TT junctions of the triad,<sup>5</sup> also known as  $\text{Ca}^{2+}$  release units (CRUs), the sites of excitation-contraction (EC) coupling. However, the precise subcellular location of STIM1-Orai1 SOCE complexes in skeletal muscle has not yet been unequivocally identified. Recent breakthroughs. Here we show by electron microscopy (EM) that prolonged muscle activity drives the formation of previously unidentified intracellular junctions between the SR and extensions of the TTs membrane. The activity-dependent formation of these unique SR-TT junctions reflects a striking and unexpected remodeling of the existing sarcotubular system at the I-band of the sarcomere. Using immunohistochemistry and immunogold labeling for EM we demonstrate that these junctions contain the molecular machinery known to mediate SOCE: STIM1 in the SR and Orai1 channels, which move into the I band as a result of the elongation of existing TTs. Thus, these newly formed junctions are referred to as  $\text{Ca}^{2+}$  Entry Units (CEUs), the first new, molecularly-defined subcellular structure in skeletal muscle in over 30 years. We propose that CEUs: a) play a fundamental role in coordinating STIM1-Orai1 coupling in muscle, b) represent the structural framework for SOCE providing an ideal  $\text{Ca}^{2+}$  entry pathway to refill SR stores, and c) plays a key role during repetitive stimulation.

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## CIR-Myo - Lecture 4

### Deep Brain Stimulation in Parkinson and dystonia

Angelo Antonini

Parkinson and Movement Disorders Unit, IRCCS Hospital San Camillo, Venice & 1st Neurology Clinic, University Hospital of Padua, Italy

E-mail: "Angelo Antonini" [angelo3000@yahoo.com](mailto:angelo3000@yahoo.com)

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) or globus pallidus internus (GPI) is now an established, safe and effective treatment option for Parkinson's disease (PD), used by more than 125,000 patients worldwide. Solid scientific evidence indicates that it can significantly alleviate motor disability, levodopa-induced complications and improve a patient's overall quality of life. The indications for DBS have expanded in recent years, including earlier application in Parkinson patients, use in other motor disorders such as dystonia and essential tremor, and the potential for use in intractable epilepsy and psychiatric disorders, for example.

In Parkinson patients DBS produces a marked improvement in motor fluctuations and dyskinesias even if evidence suggests that the reduction in motor disability is greater with medications than with STN-DBS. Benefits associated with DBS persists for many years, although disability still progresses, reflecting degeneration in non-dopaminergic sites. It is noteworthy, however, that the use of DBS is limited not only by several restrictive exclusion criteria, but also by the comparatively high risk of severe complications, such as neuropsychiatric morbidity, intracranial bleeding (which can occur in 2-8% of patients undergoing stereotactic neurosurgery), and in some cases increased mortality.

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## CIR-Myo - Lecture 5

### Link between malignant hyperthermia (MH) and environmental heat stroke (EHS): just a medical hypothesis?

Feliciano Protasi

CeSI - Center for Research on Ageing & DNICS - Dept. of Neuroscience, Imaging, and Clinical Sciences, Univ. G. d'Annunzio of Chieti, Italy

E-mail: [feliciano.protasi@unich.it](mailto:feliciano.protasi@unich.it)

EC coupling in muscle links the transverse(T)-tubule depolarization to release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR).<sup>1-2</sup> These membranes communicate in specialized structures, i.e. calcium release units (CRUs), thanks to a cross-talk between voltage-dependent  $\text{Ca}^{2+}$  channels  $\text{CaV}1.1$  (or dihydropyridine receptors, DHPRs) in the T-tubule and  $\text{Ca}^{2+}$  release channels, or ryanodine receptors type-1 (RyR1), in the SR.<sup>3-5</sup> Mutations in the gene encoding for RyR1, the SR  $\text{Ca}^{2+}$  release channel, underlie debilitating, life-threatening muscle disorders such as central core disease (CCD) and malignant hyperthermia (MH). To date, MH is only seen as a clinical syndrome in which genetically predisposed individuals respond to volatile anesthetics in the operating room with potentially lethal episodes characterized by elevations in body temperature and rhabdomyolysis of skeletal muscle fibers. However, virtually identical over-heating episodes have been reported in individuals also after exposure to environmental heat, physical exertion, or even during febrile illness. The life-threatening nature of EHS underscore the critical need for a deeper mechanistic understanding of these syndromes and for the development of new and effective



treatments. Specific Gaps of Knowledge. A) Mutations in RYR1 have been found in many, but not all, MH cases suggesting the potential involvement of additional genes in the pathogenesis of this syndrome. B) The relationship between classic MH and over-heating episodes triggered by different stressors (heat, exertion, fever, etc.) is not yet widely recognized. C) The cascade of molecular mechanisms that from SR  $\text{Ca}^{2+}$  leak leads to rhabdomyolysis of muscle fibers are still unclear and needs to be fully elucidated. Recent breakthroughs. In the last years, thanks to the support of Telethon (GGP08153 and GGP13213), we have moved significant steps forward. We have demonstrated in animal models that: A) MH episodes can result not only from mutations in RYR1, but also from mutations in proteins that interact with RYR1 (such as Calsequestrin-1, CASQ1); B) the mechanisms underlying hyperthermic episodes triggered by anesthetics and by heat and exertion are virtually identical, suggesting that these syndromes could be possibly treated/prevented using similar treatments; C) during lethal MH/EHS crises  $\text{Ca}^{2+}$  leak from intracellular stores results in a feed-forward mechanism mediated by excessive production of oxidative species of oxygen and nitrogen (ROS and RNS), which eventually will lead to depletion of the SR and to massive activation of Store Operated  $\text{Ca}^{2+}$  Entry (SOCE).

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## Workshop "Mobility in elderly": Molecular approaches – H Kern, A Musarò, Chairs

### Insights into the oxidative stress-mediated sarcopenia and dismantlement of NMJ

Antonio Musarò

Institute Pasteur Cenci Bolognietti, Unit of Histology and Medical Embryology, IIM, Sapienza University of Rome, Italy

E-mail: [antonio.musaro@uniroma1.it](mailto:antonio.musaro@uniroma1.it)

A crucial system severely affected in different pathological conditions is the antioxidative defense, leading to accumulation of ROS. The discovery that the anti-oxidant status decreases with age and it is affected in several pathological conditions, such as disuses, chronic fatigue syndrome, cancer, muscular dystrophy and amyotrophic lateral sclerosis (ALS), has placed oxidative stress as a central mechanism in the pathogenesis of these diseases. A critical aspect underlying the mechanisms of age-related muscle loss is the reduction in the number of nerve terminals, fragmentation of the neuromuscular junctions (NMJs), and a decrease in neurotransmitter release. However, considering that skeletal muscles in one of the tissues that generate considerable ROS, an important issue to address is whether a selective alteration of oxidative stress balance in skeletal muscle is sufficient to induce these alterations. Preliminary results demonstrate that muscle specific accumulation of oxidative stress induces mitochondria dysfunction/alterations and triggers NMJs dismantlement, associated with higher rate of

Acetylcholine Receptor (AChR) turnover and morphological alterations of the pre-synaptic neuromuscular endplate. We also defined the potential molecular mechanisms that mediate the toxic effects of oxidative stress and NMJs dismantlement.

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### Muscle trophism and mitochondria dynamics in ageing human skeletal muscle trained with Electrical Stimulation

Sandra Zampieri (1,2), Cristina Mammucari (1), Marco Sandri (1, 3), Vanina Romanello (3), Antonio Musarò (4), Laura Barberi (4), Feliciano Protasi (5), Laura Pietrangelo (5), Aurora Fusella (5), Stefan Loeffler (2), Jan Cevka (6), Nejc Sarbon (7), Rosario Rizzuto (1), Ugo Carraro (8), Helmut Kern (2)

(1) Department of Biomedical Sciences, Padova, Italy; (2) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Vienna, Austria; (3) Venetian Institute of Molecular Medicine, Dulbecco Telethon Institute; (4) DAHFMU-Unit of Histology and Medical Embryology, IIM, Sapienza University of Rome, Italy; (5) CeSI - Center for Research on Aging & DNICS, Department of Neuroscience, Imaging and Clinical Sciences, University G. d'Annunzio, Chieti, Italy; (6) Faculty of Physical Education and Sport, Comenius University, Bratislava, Slovakia; (7) University of Primorska, Science and Research Centre, Institute for Kinesiological Research, Koper, Slovenia; (8) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy

E-mail: [sanzamp@unipd.it](mailto:sanzamp@unipd.it)

Physical exercise is known to have beneficial effects on muscle trophism and force production modulating signaling pathways involved in fiber type and muscle growth also via intracellular  $\text{Ca}^{2+}$  and inducing specific mitochondrial adaptations. Several evidences both in vitro and in vivo, have demonstrated that during muscle contraction,  $\text{Ca}^{2+}$  concentration in the mitochondrial matrix is increased. Importantly, alterations of mitochondrial  $\text{Ca}^{2+}$  homeostasis controlled by mitochondrial calcium uniporter (MCU) has been recently shown to regulate muscle mass in vivo in mice. In skeletal muscle, mitochondria exist as dynamic network that is continuously remodeled through fusion and fission phenomena that are important for maintenance of functional mitochondria. In particular, fission occurs upon the recruitment of dynamin-related protein 1 (DRP1), while fusion is controlled by mitofusins (MFN) 1 and 2 and by optic atrophy 1 (OPA1). OPA1 also regulates mitochondrial adaptations to bioenergetic conditions at the level of inner membrane ultrastructure and remodeling of mitochondrial cristae, controlling muscle atrophy and mitochondria respiratory efficiency. It has been shown that the decrease of muscle mass and strength observed in age-related Sarcopenia is linked to abnormalities of mitochondrial morphology, number and function. In the present study we intended to investigate the effects of 9 weeks strength training by neuromuscular electrical stimulation (ES) in comparison to voluntary leg press (LP) in sedentary 70yrs old subjects on mitochondria dynamics, MCU and mitochondria respiratory chain enzymes modulation. Our results show that ES mediated muscle structural and functional improvements are linked to signaling pathways related to muscle mass regulation and enhanced MCU, and COX IV respiratory chain enzyme. Electron Microscopy ultrastructural analyses showed remodelling of mitochondrial apparatus as a consequence of fusion phenomena





that is consistent with adaptation to physical exercise and with increased OPA1 protein expression levels as documented by WB analyses. LP training showed moderate effects both at structural and functional level, with no impact on mitochondrial dynamics, that are consistent with a milder protocol of training in comparison to neuromuscular ES. Altogether these results indicate that the ES-dependent beneficial effects on muscle mass and force are associated with changes in mitochondrial-related proteins involved in Ca<sup>2+</sup> homeostasis and mitochondrial shape, providing new targets to develop therapeutic strategies counteracting Sarcopenia and promoting healthy ageing.

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### Ectopic expression of *Merg1a* in skeletal muscle of mice modulates NFκB activity

Sohaib Hameed (1), Kevin Bradley (1), Luke Anderson (1), Chase Latour (1), Nicole Dethrow (1), Emi Hayashi Park (1), Mariam Hashmi (1), Sandra Zampieri (2,3), Ugo Carraro (4), Amber Pond (1)

#Southern Illinois University School of Medicine, Carbondale, IL, US; (2) Department of Biomedical Sciences, Padova, Italy; (3) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Vienna, Austria; (4) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy

E-mail: "Amber Pond" [apond@siumed.edu](mailto:apond@siumed.edu)

Skeletal muscle atrophy is a debilitating loss of muscle mass (resulting from decreased myofiber size) and strength that normally occurs during aging, muscle disuse / inactivity, neuropathies / myopathies and with other pathological diseases. The ether-a-gogo related gene (*ERG1a*) is a K<sup>+</sup> channel that is up-regulated in atrophying gastrocnemius muscles of both unweighted and cachectic mice.<sup>1</sup> Ectopic expression of mouse *erg1a* (*Merg1a*) in mouse muscle increases ubiquitin proteasome activity by up-regulation of at least one known E3 ligase, MuRF1.<sup>2</sup> Because *Murf1*

expression is up-regulated by increased NFκB activity<sup>3</sup>, we hypothesized that ectopic expression of *Merg1a* would increase NFκB activity and lead to increased *Murf1*. To test this, we electro-transferred plasmid encoding an NFκB firefly luciferase (FFL) reporter and a second plasmid encoding *Renilla* luciferase (*Ren*) into mouse gastrocnemius muscles. We added a *Merg1a* plasmid in the left leg while adding a control plasmid in the right. We then harvested muscle on days 0-7 and assayed for both FFL and *Ren* activities and used the FFL to *Ren* ratio as a measure of NFκB activity (to correct for differences in transfection efficiency). Surprisingly, the muscles expressing *Merg1a* showed a decreased NFκB activity when compared to the controls. Thus we hypothesized that there may be a factor present in a physiological model of atrophy that would cause *MERG1a* to modulate NFκB activity differently. Because sciatic nerve transection does not produce an increase in *Merg1a* expression, but it increases NFκB activity<sup>3</sup>, we repeated our electro-transfer with mice and then denervated both legs 18-24 hours later. We harvested muscles at days 0, 2, 4, 7 and 10 post-denervation. The *Merg1a* treated muscle still experienced a decrease in NFκB activity. Thus, we conclude that ectopic expression of *Merg1a* modulates NFκB activity in both innervated and denervated skeletal muscle. Future studies will include efforts to determine if this finding is truly physiological and, if so, then by what mechanism does *MERG1a* affect NFκB activity.

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### Molecular adaptation of MHC and Ca<sup>2+</sup>-handling proteins of human skeletal muscle to aging and FES: an *in situ* study

Simone Mosole (1,2), Sandra Zampieri (1,2), Sandra Furlan(3), Barbara Ravara (1,2), Hanna Fruhmman (2), Stefan Löffler (2), Michael Vogelauer (4), Helmut Kern (2,4), Ugo Carraro (5), Pompeo Volpe (1), Alessandra Nori (1)

(1) Laboratory of Translational Myology of the Interdepartmental Research Center of Myology, Department of Biomedical Science, University of Padova; (2) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Vienna, Austria; (3) Institute of Neuroscience Consiglio Nazionale delle Ricerche, Padova, Italy; (4) Institute of Physical Medicine and Rehabilitation, Wilhelminenspital, Vienna, Austria; (5) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy

E-mail: [simone.mosole@studenti.unipd.it](mailto:simone.mosole@studenti.unipd.it)

Physical activity plays an important role in preventing chronic disease and muscle degeneration in adults and the elderly persons.<sup>1</sup> Age-related changes in skeletal muscle innervation, independent of patent peripheral neuropathies, are known to contribute to the decline in quality of life often reported in older population.<sup>2</sup> The changes and the mechanism(s) by which they occur are not well understood. We had the opportunity to examine the effects of lifelong high-level physical activity comparing cohorts of young



adults and septuagenarians either sedentary or recreational sportsmen, collecting what is, in our opinion, strong evidence that aging atrophy is, at least in part, a result of progressive denervation that can be counter-acted by lifelong high-level exercise.<sup>3</sup> We used immunolabeling methods to analyze the fiber type composition of muscle biopsies. Our main results demonstrate that biopsies from: 1. young men seldom contain denervated ( $0.2 \pm 0.5\%$ ), or transforming muscle fibers ( $0.5 \pm 0.6\%$ ); 2. sedentary seniors contain both denervated ( $2.6 \pm 1.9\%$ ), coexpressing myofibers ( $1.8 \pm 1.7\%$ ) and a few reinnervated clustered myofibers of the fast type ( $3.0 \pm 4.7\%$ ); and 3. senior sportsmen present with a larger percentage of healthy, slow myofibers (up to  $68.5 \pm 14.1\%$ ), that appear mainly clustered in slow fiber-type groupings ( $7.9 \pm 7.4\%$ ).<sup>4,5</sup> Data analysis reveals that there was no difference between the athletic and sedentary senior groups in terms of their very low percentages of muscle fibers co-expressing fast and slow MHCs ( $0.6 \pm 0.6\%$ ), suggesting that lifelong exercise does not induce motor unit transformation. On the other hand the recreational sportsmen had both considerably higher percentages of slow-type myofibers and greater numbers of slow fiber-type groupings, providing sound evidence that lifelong cycles of denervation/reinnervation occurred. It appears that lifelong exercise protects muscle function by saving otherwise lost muscle fibers through reinnervation by different, mainly slow, motor axons.<sup>6</sup>

On the other hand, volitional exercise is not always feasible or people are reluctant to do it and other strategies should be applied such as Functional Electrical Stimulation (FES).<sup>7</sup> This study shows the effects *in situ* of FES in human *Vastus Lateralis* (VL) muscle of sedentary elderly people, in particular on the key process of  $\text{Ca}^{2+}$  uptake and release and related control mechanisms that are essential in muscle adaptation. Through immunofluorescence analysis of muscle cryosections, a huge increment of NFAT positive nuclei was found after treatment (from 3% to 60%); moreover an increment of P-CamKII was observed by western blotting analysis. These findings indicate that FES activate the CaM-dependent phosphatase signaling (known to be involved in muscle plasticity). Muscle total homogenates were obtained from biopsies performed before and after completing a nine weeks FES treatment on a group of volunteers and Calsequestrin (CASQ), SERCA, Sarcoplumenin, protein expression was determined by Western blot. After FES significant increase of SERCA2 and Sarcoplumenin and decrease of CASQ1 were observed. Immunofluorescence analysis were also performed to localize *in situ* MHCII/SERCA2 co-expressing muscle fibers, an interesting tool to identify subpopulation of muscle fibers involved in muscle adaptation. The overall results indicate that the applied FES protocol, simulating a motoneuron slow-type firing pattern, potentiates  $\text{Ca}^{2+}$  uptake and storage in skeletal muscle fibers validating at molecular level the FES strategy as a safe and effective rehabilitation strategy in elderly persons.

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### Tat-MyoD fused proteins, together with C2C12 conditioned medium, are able to induce equine adult mesenchymal stem cells towards the myogenic fate

Chiara Gomiero (1), Tiziana Martinello T(1), Alessandro Negro (2), OhaDTopel (3), Roberta Sacchetto (1), Marco Patruno (1)

(1) Department of Comparative Biomedicine and Food Science, University of Padova, Italy; (2) Department of Biomedical Sciences, University of Padova, Italy; (3) VTH -Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Israel

E-mail: [marco.pat@unipd.it](mailto:marco.pat@unipd.it)

The Tat protein is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. This ability appears particularly interesting to induce tissue-specific differentiation when the Tat protein is associated to transcription factors. In the present work the potential of the complex Tat-MyoD in inducing equine peripheral blood mesenchymal stromal cells (PB-MSCs) towards the myogenic fate, was evaluated. Results showed that the internalization process of Tat-MyoD needs the absence of serum and the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of Tat-MyoD only was not sufficient to induce myogenesis and, therefore, in order to achieve the myogenic differentiation of PB-MSCs, conditioned medium was added. The latter was obtained coculturing PB-MSCs with C2C12 without a direct contact. These results suggest that TAT-mediated protein transduction system, if supported by conditioned medium, might represents a useful methodology to induce myoblasts differentiation.

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## Mobility impairments in aging and myopathies

Stefano Masiero, Francesco Piccione, Chairs

### Decline of power developed by skeletal muscles based on world records of Master athletes from 30 to 110 years

Paolo Gava (1), Ugo Carraro (2)

(1) Laboratory of Translational Myology of the Interdepartmental Research Center of Myology, Department of Biomedical Science, University of Padua; (2) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy

E-mail: "Paolo Gava" [Paologavastra@alice.it](mailto:Paologavastra@alice.it)

Both strength and power developed by human skeletal muscles decline with increasing age.<sup>1,2</sup> The athletic world records of the Master athletes of age ranging from 35 years to 100 years are an excellent proof of such decline in every track and field competition. The world record performances of running, jumping and throwing events can be transformed into dimensionless parameters proportional to the power developed in the trials. Such parameter ranges from 1 for the Senior world record (i.e. the maximum human performance) to lower values for the Master athletes of increasing age down to 0 for a null performance.<sup>3,4</sup> With this procedure the declines of the power parameter with increasing age can be analysed and compared: the trend-lines start to decline very close to the age of 30 years and arrive to 0 around the age of 110 years for every athletic discipline (running, jumping and throwing).<sup>5</sup> The comparison of the various trend-lines show significantly different rates of decline. Each declining trend-line reaches a "critical" threshold at different ages for the running, jumping and throwing activities. Such thresholds indicate different age limits for most of everyday tasks: walking, climbing stairs and lifting weights above a table. The decline of the Master world records, transformed into a dimensionless power parameter declining from 1 toward 0 with increasing age is the decline of the power developed by each one of us, starting from 1 in our youthful age and declining toward lower values with increasing age. There are no reason, for each one of us, to decline differently from the world record-men, provided that each of us remains in stable fitness condition without disabling pathologies.

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## Electrical Stimulation in neuromuscular impairments: an educational case report

Andrea Marcante (1), Paolo Gargiulo (2), Ugo Carraro (1), Francesco Piccione (1)

1 IRCCS Fondazione Ospedale San Camillo, Venezia Lido, Italy; (2) Institute for Biomedical and Neural Engineering/ Biomedical Technology Centre Reykjavik University & Landspítali Reykjavik, Iceland

E-mail: [andrea.marcante@ospedalesancamillo.net](mailto:andrea.marcante@ospedalesancamillo.net)

We present a case report of atypical amyotrophic neuralgia of the suprascapular nerve with isolated denervation atrophy of rotator cuff muscles and related biomechanics impairments of the shoulder. The patient, lamenting yearly-long unsatisfactory results of standard clinical physiotherapy, after a baseline re-evaluation at our hospital, have been treated for the last seven months with an additional personalized home-based Electrical Stimulation protocol using triangular currents for denervated muscles. At the end of the follow up we observed clinical, radiological (False-color CT)<sup>1</sup> and neurophysiological (needle-EMG)<sup>2</sup> improvements. The case report provides the opportunity to discuss a rehabilitative pathway for diagnostics and rehabilitation of patients suffering of peripheral denervation, a condition that is still a challenge for clinicians.

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## Gut inflammation and chronic low back pain: physiopathology

Sergio Veneziani, Christian Testa, Claudio Carlo Castelli

Ortopedia e Traumatologia e Dipartimento di Emergenza-Urgenza, Azienda Ospedaliera "Papa Giovanni XXIII", Bergamo, Italy

E-mail: [sveneziani@hpg23.it](mailto:sveneziani@hpg23.it)

The Chronic low back pain (CLBP) is a disabling condition affecting a majority of people of the western countries. It also deeply affects the quality of life as it is often linked to multidimensional disturbances such as poor sleep, mood disorders, chronic fatigue and joint pain. There is no condition with higher social and economic expenses. It has been reported that only a minority of patients with gut inflammation suffers from intestinal symptoms. In a previous paper it was proposed that gastrointestinal disturbances, beyond mechanical issues, could be overlooked in the management of these patients. Dietary changes were successful in the positive resolution of the described clinical case. Here we further test this hypothesis. We measured on 5 subjects specific parameters related to gastrointestinal and digestive physiology that have been associated with metabolic and immuno-related pathological conditions. Inflammation in the gut can lead to altered mucosa permeability. The entrance in the blood stream of abnormal molecules activates the immune system in a cascade of events affecting remote systems and possibly the integrity of structures like the neuromuscular junction or the pathways of energy production. Conditions that are





currently managed by orthopaedists, rheumatologists and neurologists could benefit from a screening of the gastrointestinal functionality.

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### Novel insights into skeletal muscle function by mechanomyography in health and disease

Fabio Esposito

Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy

E-mail: [fabio.esposito@unimi.it](mailto:fabio.esposito@unimi.it)

In the last decades, a growing body of literature focused on the use of mechanomyography (MMG) as a means to study non-invasively skeletal muscle mechanical activity. MMG signal is detectable at the skin surface during the dimensional changes of active muscle fibres that generate pressure waves due to voluntary or evoked contractions.<sup>1,2</sup> A novel application is the use of an electromyographic (EMG), MMG, and force (F) signals combined approach as a tool to partition the electrochemical and mechanical events underpinning the electromechanical delay during muscle contraction (EMD) and relaxation (R-EMD).<sup>3,4</sup> This approach has been utilized to evaluate the changes in the electrochemical and the mechanical components of EMD and R-EMD under several physiological conditions (local fatigue, muscle temperature manipulation and muscle-tendon unit stretching). Under all these circumstances, the approach presented a high reliability and sensitivity. Myotonic dystrophy type 1, the most frequent form of inherited muscular dystrophy<sup>5</sup> involves a broad spectrum of systemic complications. The main features at the skeletal muscle level are muscle weakness and grip and percussion myotonia. Distal muscles are generally more compromised than the proximal ones. In clinical settings, muscle weakness and myotonia are usually determined on patients with DM1 qualitatively or semiquantitatively by the Medical Research Council scale, by dynamometry, and/or by physician's handgrip evaluation. Hence, a valid, non-invasive, and reliable tool to assess the degree of muscle dysfunction in DM1 could be of great interest for clinical trials involving new therapies. Therefore, the aims of the study were: (i) to assess the reliability and sensitivity of the measurement of the electromechanical delay components during both contraction and relaxation in patients with DM1; and (ii)

to evaluate and discuss possible differences in delay components' duration between patients with DM1 and healthy, age-matched controls (HC). EMD and R-EMD electrochemical and mechanical components duration and reliability of the measurements were investigated during skeletal muscle contraction and relaxation in a group of patients with DM1 (n = 13) and in healthy controls (n = 13). EMG, MMG, and F were recorded from the tibialis anterior (distal muscle) and vastus lateralis (proximal muscle) muscles during maximum voluntary and electrically-evoked isometric contractions. The electrochemical and mechanical components of the electromechanical delay during muscle contraction and relaxation were calculated off-line. Maximum strength was significantly lower in DM1 than in controls under both experimental conditions. All electrochemical and mechanical components were significantly longer in DM1 in both muscles. Measurement reliability was very high in both DM1 and controls. The high reliability of the measurements and the differences between DM1 patients and controls suggest that the EMG, MMG, and force combined approach could be utilized as a valid tool to assess the level of neuromuscular dysfunction in this pathology, and to follow the efficacy of pharmacological or nonpharmacological interventions.

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### CIR-myo - Lecture 6

#### Muscle and bone assessed by Color CT, gait analysis and EMG

Kyle J. Edmunds(1), Iris Árnadóttir (1), Magnus K. Gíslason (1), Ugo Carraro (2), Paolo Gargiulo (1,3)

(1) Institute for Biomedical and Neural Engineering, Reykjavík University, Reykjavík, Iceland; (2) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy; (3) Department of Rehabilitation, Landspítali, Reykjavík, Iceland

E-mail: [paologargiulo@landspitali.is](mailto:paologargiulo@landspitali.is)

The optimum metric for assessing changes in skeletal muscle quality remains debated. Identifying a novel quantitative method for muscle assessment in this regard would allow for the generalizability of such studies to clinical practice and therefore aid in the indication of compensatory targets for clinical intervention.<sup>1-3</sup> While there is much extant literature reporting the use of average HU values to investigate muscle quality and its utility as a comorbidity index, no studies have yet to utilize the entire radiodensitometric distribution.



The increasing prevalence of sarcopenic and cachexic muscular degeneration necessitates the establishment of a robust quantitative muscle assessment methodology.<sup>4</sup> Herein, we hypothesize that rigorously quantifying entire HU distributions can elicit much more information regarding muscle quality than extant methods that, to date, only utilize average HU attenuation values. This study reports the development and use of this method, wherein we assess upper leg muscle quality utilizing nonlinear trimodal regression analysis with radiodensitometric distributions from computed tomography (CT) scans of a healthy young adult, a healthy elderly subject, and an SCI patient with complete lower motor neuron denervation. Results from this assessment highlight the utility of utilizing entire HU attenuation value distributions and identify novel parameters from these analyses that could provide further insight into how muscular degradation can be optimally quantified.

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## SATURDAY April 16, 2016

Hotel Augustus, Viale Stazione 150, Montegrotto Terme (Padova), Italy

### CIR-Myo-Lecture 7

The RISE Project: roots, outcomes, byproducts  
Helmut Kern, Vienna, Austria

Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, and Dept. of Physical Medicine and Rehabilitation, Wilhelminenspital Wien, Austria  
E-mail: helmut.kern@wienkav.at

During the last decade we contributed to rehabilitation in aging studying effects of physical exercise induced by Functional Electrical Stimulation (FES) in the special case of Spinal Cord Injury patients affected by complete injury of the Conus Cauda, a syndrome in which the denervated leg muscles are fully disconnected from the nervous system. Denervated human muscles become unexcitable with commercial electrical stimulators and undergo ultra structural disorganization within a few months from SCI, while severe atrophy with nuclear clumping and fibro-fatty degeneration appear within 3 and 6 years, respectively.<sup>1-4</sup> To counteract these progressive changes a novel therapy concept for paraplegic patients with complete lower motor neuron denervation of the lower extremity was developed in

Vienna: home-based functional electrical stimulation of long-term denervated muscles (h-b FES). New electrodes and a safe stimulator for h-b FES have been designed to reverse severe atrophy by delivering high-intensity (up to 2,4 J) and long- duration impulses (up to 150 ms) able to elicit contractions of denervated skeletal muscle fibers in absence of nerves.<sup>5,6</sup> Specific clinical assessments and trainings were developed at the Wilhelminenspital Wien, Austria,<sup>7</sup> based on sound evidence from animal experiments.<sup>8</sup> Main results of the clinical study on patients which completed the 2-year h-b FES training were: 1. significant increase of muscle mass and of myofiber size, with striking improvements of the ultra- structural organization; 2. recovery of tetanic contractility with significant increase in muscle force output during electrical stimulation; 3. capacity to perform FES-assisted stand-up and stepping-in-place exercise.<sup>9-12</sup>

The study demonstrated that h-b FES of permanent denervated muscle is an effective home therapy that results in rescue of muscle mass, function and perfusion. Additional benefits are improved leg cosmetic appearance and enhanced cushioning effect for seating.

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## MED\*EL Workshop Functional Rejuvenation in Aging

Werner Lindenthaler, Winfried Mayr, Chairs

### The effects of chronic electrical muscle stimulation (EMS) following nerve injury on time to reinnervation and appropriate reinnervation of muscular targets

**Mike P Willand (1), Joseph Catapano (1), Jennifer J Zhang (1), Hermann Lanmueller (2), Ewald Unger (2), Martin Schmoll (2,5), Jon Cheetham (3), Jonathan Norm Ducharme (3), Marta Cercone (3), David Zealear (4), Yike Li (4), Jonathan C. Jarvis (5), Gregory Borschel (1), Tessa Gordon (1)**

(1) Division of Plastic and Reconstructive Surgery, Department of Surgery, The Hospital for Sick Children, Toronto, ON, Canada; (2) Center for Medical Physics and Biomedical Engineering, Medical University Vienna, Vienna, Austria; (3) College of Veterinary Medicine, Cornell University, Ithaca, NY, USA; (4) Department of Otolaryngology, Vanderbilt University, Nashville, TN, USA; (5) School of Sports and Exercise Sciences, Liverpool John Moores University, Liverpool, UK.

E-mail: [mike.willand@gmail.com](mailto:mike.willand@gmail.com)

Functional recovery after peripheral nerve injury is reduced when axon growth is misdirected to reinnervate muscles other than their original targets. 1-3 Here we review the effects of chronic electrical muscle stimulation (EMS) following peripheral nerve injury in rat, canine, and equine models of peripheral nerve injury. Specifically, we examine whether EMS accelerates reinnervation of muscular targets and if these targets are appropriately reinnervated by their original axons following nerve injury. Methods: In the Sprague Dawley rat, the lateral gastrocnemius nerve was transected and immediately repaired. The soleus muscle was implanted with electrodes and connected to a mini stimulator implanted intra-abdominally. Muscles were stimulated daily using a 12-hour day time pattern of a 10 second burst of 20 Hz once per hour followed by a 12-hour night time pattern of 20 Hz (10 seconds on, 20 seconds off). This stimulation pattern was delivered for 2 months. Appropriate reinnervation of the soleus muscle was assessed using retrograde labeling of the soleus nerve. Functional recovery was assessed by measuring isometric soleus muscle forces. In the dog, the recurrent laryngeal nerve was transected bilaterally and immediately repaired. Electrodes were implanted to stimulate the posterior cricoid arytenoid (PCA) muscles bilaterally. Muscles were stimulated continuously using either a 10 or 40 Hz pulse train for ninety days. Appropriate reinnervation was measured using electromyography methods. Functional recovery was assessed using a treadmill exercise test and vocal fold movement during hypercapnia. In the horse, the right recurrent laryngeal nerve was injured using a stainless steel probe pre-chilled in liquid nitrogen and placed on the nerve for two minutes. Electrodes were implanted into the right PCA muscle and connected to an implanted stimulator. Muscles were stimulated for one hour once every 12 hours at 22 Hz

(3.5 seconds on, 6.5 seconds off) for twenty weeks. Functional recovery was assessed using a treadmill exercise test and examining arytenoid abduction and tracheal inspiratory pressure endoscopically. Results: Retrograde labeling in the rat, demonstrated that EMS of the soleus muscle had no effect on directing the original soleus neurons back to reinnervate the muscle following nerve injury and repair. Muscle twitch forces were significantly greater, however, tetanic forces were not different whether the muscle was stimulated or not. In the dog, progressive addition of samples to the study showed that exercise tolerance and glottal area following hypercapnia was maximal in dogs that received PCA stimulation at 10 Hz. 4 EMG measurements in 3 dogs suggest that PCA muscles stimulated at 10 Hz were preferentially reinnervated by their original motoneurons whereas those muscles stimulated at 40 Hz or were unstimulated had random reinnervation. Horses that had the PCA muscle stimulated had improved function as demonstrated by lower negative tracheal inspiratory pressures. These improvements occurred sooner after injury and at lower exercise intensities than horses that did not have the PCA muscle stimulated. However, functional recovery returned to near baseline in all horses suggesting that the original nerve injury was not severe enough. Conclusions: Despite stimulating the soleus muscle in rats using a pattern resembling natural activity before and during the time of reinnervation, EMS did not encourage the original motoneurons that were connected to the stimulated muscle to return. However, in the dog, 10 Hz stimulation promoted selective reinnervation. One limitation in the dog study is the small sample size which needs to be expanded to provide adequate statistical power. In the horse, EMS enhanced the speed of functional recovery despite a nerve injury that was not as severe as one in both dogs and rats. Nevertheless, in all animal models stimulation did not negatively impact functional recovery with muscle forces in the rat being higher with stimulation and dynamic airway measurements being enhanced in both dogs and horses that had their muscles stimulated following nerve injury.

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### Establishing a hypertrophy model in rats using 'SpillOver' stimulation to cause co-contraction

**Jonathan C. Jarvis (1), Martin Schmoll (1,2), Manfred Bijak (2), Michael Haller (2), Ewald Unger (2), Hazel Sutherland (3), Hermann Lanmueller (2)**

(1) School of Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UK; (2) Center of Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria; (3) Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, UK Research Institute for





Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UK.

E-mail: "Jarvis, Jonathan" [J.C.Jarvis@ljmu.ac.uk](mailto:J.C.Jarvis@ljmu.ac.uk)

The cellular mechanisms underpinning the maintenance, gain and loss of muscle mass are of great interest at present, given the popularity of bodybuilding, the potential for increased muscle metabolism to reduce the damage caused by diabetes and insulin resistance, and the key role of muscle function associated with the decline in mobility and well-being associated with ageing. There are many model systems in which to make experimental investigations of muscle hypertrophy in rodents.<sup>1-9</sup> It is generally considered that increased average force generation (loading) is important, although hypertrophy can be achieved in some muscles by pharmaceutical agonism of the androgen receptor family, or by genetic manipulation of, for example, the response to IGF. Models have included removal<sup>1,2,3</sup> or denervation of agonists to generate constant overload, various training modalities such as squats,<sup>3</sup> lifting,<sup>4,5</sup> or jumping for a food reward, and treadmill or ladder climbing exercise,<sup>6</sup> sometimes with added weight<sup>7</sup> to increase the muscular effort. We have designed a hypertrophy model using programmed exercise by stimulating agonists and antagonists simultaneously. In the rat hind limb, the dorsiflexor muscles that lift the foot are supplied by the common peroneal nerve whereas the plantarflexor muscles are supplied by the tibial nerve. The plantarflexors are the larger and stronger group so it is possible to generate loaded contractions of the dorsiflexors by activating them fully at the same time as a partial activation of the plantarflexors. We have achieved this with a single channel implant by careful positioning of the electrodes with the cathode under the common peroneal nerve and the anode near to the tibial nerve as it runs on the proximal posterior surface of the gastrocnemius muscle. The key to success in this model is the ability to adjust remotely the stimulating current and to choose a stimulation pattern that generates high force contractions with minimal disturbance to the subject. Using the new miniVStim device developed between Vienna and Liverpool we are able to programme an 'adaptation' pattern so that the subject is accustomed to the sensation of muscle activation at a low level before the loaded contractions are made. With stimulation in one session per day of 5 sets of 10 repetitions at 100Hz (2s ON 2s OFF) and 2.5 minutes between sets, we have achieved hypertrophy of the tibialis anterior muscle giving an increase in wet weight of between 11.5 and 13.7% in 5 rats over 4 weeks. We will use this model to investigate further the sensitivity to hypertrophy of the various fibre types and the cellular pathways that are activated in this response.

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### Investigating Energy Efficiency for different Pulse shapes and electrode arrangements to activate motor neurones

Martin Schmoll (1,2), Jonathan C. Jarvis (1)

(1) School of Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UK; (2) Center of Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria.

E-mail: [martin.schmoll@meduniwien.ac.at](mailto:martin.schmoll@meduniwien.ac.at)

One of the main determinants of the size of neural implantable pulse generators is the size of the battery. The challenge for engineers is to design devices that are small in volume whilst fulfilling their stimulation task as long as possible. Efficient stimulation methods are crucial for their success. Wongsarnpigoon pointed out three different types of efficiency relating to nerve activation.<sup>1</sup> A "charge-efficient" stimulation has the positive effect of reducing tissue damage. As the battery size is proportional to the maximal instantaneous power required, "power-efficient" stimulation could reduce battery-size and therefore the overall size of an implant. An "energy-efficient" stimulation on the other hand, increases the battery lifetime. We have compared different waveforms according to their "energy-efficiency". Six different waveforms have been investigated (rectangular monophasic, rectangular biphasic, rectangular biphasic with interphase gap, gaussian biphasic, exponential biphasic, asymmetric rectangular biphasic with interphase gap) to clarify some of the potentially useful efficiencies noted in other studies.<sup>2-7</sup> Another interesting aspect that has been investigated in our latest experiments, is a comparison between monopolar and bipolar stimulation, and some investigation of the transition between the monopolar and the bipolar configuration in terms of stimulation efficiency. For bipolar stimulation, two stainless-steel loop electrodes were placed under the common peroneal nerve of rats under buprenorphine/isoflurane anaesthesia. In the monopolar situation the anodal electrode was a hypodermic needle placed under the skin more than 50mm away from the cathode. The cathode was the same in both cases. The isometric force produced at optimal length by the extensor-digitorum-longus muscle was measured using a load-cell. Our results showed a noticeable difference between monopolar and bipolar stimulation. Monopolar stimulation showed generally higher energy levels than bipolar stimulation to activate the motor neurones of the common peroneal nerve. While the introduction of an interphase-gap increased the threshold current, where the force was 10% of the control-force, in the bipolar case, a reduction was observed in the monopolar situation. In previous bipolar experiments we found an increased energy requirement for threshold activation with asymmetric waveforms while the monopolar stimulation again showed a reduction. The results show similar effects as described in literature when using monopolar stimulation. Nevertheless we achieved different results for the bipolar stimulation regime. It is therefore important to bear these differences in mind, when



designing electrodes and patterns of stimulation to improve stimulation efficiency.

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### MiniVStim18B: A highly configurable battery powered FES implant for long-term implantation in small animals

Manfred Bijak (1), Ewald Unger (1), Michael Haller (1), Martin Schmoll (1,2), Jonathan C. Jarvis (2), Hermann Lanmüller (1)

Center of Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria. (2) Liverpool John Moores University, UK

E-mail: [manfred.bijak@meduniwien.ac.at](mailto:manfred.bijak@meduniwien.ac.at)

According to PubMed roughly 10% of the annually added publications in the Life Sciences describe findings obtained from animal models. Since half of these studies are done in mice and rats it can be assumed that there is a need for implantable electrical stimulators which are flexible, reliable and small enough (~1 cm<sup>3</sup>) that implantation is possible in mice. It is important that animals do not have to be isolated during stimulation periods and that they can run freely. MiniVStim 12A is a battery powered implantable electrical stimulator able to deliver constant current monophasic, rectangular pulses up to 2mA and 1ms pulse width (@1kOhm). It is easy to use because the required stimulation pattern is preprogrammed during manufacturing. On, off or different stimulation patterns can be cyclically activated with a strong magnet, also through the skin. This implant has an outer diameter of 15 mm and a volume of 1.2 cm<sup>3</sup>. MiniVStim 12B has the same mechanical dimensions but can be fully programmed via a transcutaneous bidirectional data link. Both types of implants are already successfully used in studies.<sup>1</sup> The latest generation of implants is represented by the new MiniVStim18B. It is slightly larger (22mm outer diameter, 5.3 cm<sup>3</sup>) than its predecessors but offers an 8 fold longer battery life. Additionally, it can deliver biphasic constant current pulses and extends the stimulation parameter range up to 8mA at a maximum output voltage of 10V and with a pulse width of 5ms (@1kOhm) for monophasic and 2x5ms for biphasic pulses.

Lifetime is strongly dependent on the chosen stimulation pattern. For example, monophasic stimulation with a duty cycle of 20% (20% on, 80% off time) and 2mA, 100Hz, 250µs pulse width, 1kOhm load, leads to a battery lifetime of 300 days and when stimulating with 8mA life time comes to 70 days. Under the same circumstances except choosing stimulation frequency of 10Hz a lifetime of 1000 days and 450 days could be expected. The very low standby current consumption (<8µA) helps to increase the battery life time proportionally when stimulation is intermittently applied, like in most applications. If there is no stimulation at all, the 'shelf life' is nearly 4 years.

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### Chronic electrical stimulation in rodents: Results of a feasibility study

Michael Karbiener

ENT University Hospital Graz, Department of Phoniatrics, Medical University of Graz, Austria

E-mail: [michael.karbiener@medunigraz.at](mailto:michael.karbiener@medunigraz.at)

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### Chronic neurostimulation for treatment of age related laryngeal muscular atrophy

Marcus Gugatschka (1), Michael Karbiener (1), Justin Perkins (2), Claus Gerstenberger (1), Jonathan C. Jarvis (3), Gerhard Friedrich (1)

(1) ENT University Hospital Graz, Department of Phoniatrics, Medical University of Graz, Austria; (2). The Royal Veterinary College, Department of Clinical Sciences and Services, London, UK, 3. Faculty of Science, John Moores University, Liverpool, UK

E-mail: [Markus.Gugatschka@klinikum-graz.at](mailto:Markus.Gugatschka@klinikum-graz.at)

Muscle atrophy as part of the ageing process also affects the larynx, where it constitutes the major cause of presbyphonia. Current treatment options are mainly conservative or phonosurgically based and are far from being satisfactory. Electrical stimulation of motor neurons constitutes a promising strategy. Materials and Methods: Using aged sheep as an animal model electrical chronic stimulation of laryngeal muscles was achieved via a mini-electrode that targeted the right recurrent laryngeal nerve (RLN; unilateral stimulation). Functional electrical stimulation (FES) implants were programmed to deliver a pattern able to evoke supramaximal muscle stimulation over a period of 29 days. At the end of the study, vocalis and posterior crico-arytenoid muscles were excised and analyzed molecularly and histologically. To quantify the expression levels of genes related to distinct muscle fiber types, a real-time PCR (RT-qPCR) analysis pipeline was newly established. Results: First results showed a shift towards larger muscle fiber diameters of the stimulated side, compared to the unstimulated control side. Based on this, chronic electrical stimulation of the RLN can induce hypertrophy of the vocalis muscle even after a relatively short stimulation period of 29 days. Upcoming trials will focus on longer stimulation periods as well as more intense stimulation algorithms.

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## MED<sup>EL</sup> Workshop: ES in Neuromuscular Disorders,

Jonathan C. Jarvis, Feliciano Protasi, Chairs

### Method for selective surface stimulation of denervated muscles in the larynx

Berit Schneider-Stickler (1), Matthias Leonhard (2), Lukas Kneisz (2), Winfried Mayr (3)

(1) Medical University of Vienna, Austria; (2), Med-El, Innsbruck, Austria; (3) Center of Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria.

E-mail: [berit.schneider@meduniwien.ac.at](mailto:berit.schneider@meduniwien.ac.at)

Vocal fold paralysis is a pathological motion impairment of one vocal fold, mostly caused by laryngeal nerve damage. If the vocal fold does not reinnervate a flaccid paralysis occurs due to denervation of the vocalis muscle and its atrophy.<sup>1</sup> Patients with unilateral vocal cord paralysis suffer from hoarse and weak voice since there is always a remaining glottic gap during phonation. Today's standard treatment of unilateral paralysis includes surgical medialization through either injection augmentation or laryngoplastic framework surgery.<sup>2</sup> We want to investigate whether it is possible to selectively stimulate the denervated muscle fibers of the vocalis without causing pain or excitation of sensory nerve fibers or activation of innervated muscles in the neck region. The goal is 1. a verification of functionality for screening and 2. a strengthening and increase in total volume of the target muscle on order to improve voice quality in patients with unilateral paralysis. In combination with voice therapy also electrical stimulation of laryngeal muscles has already been used in order to achieve hypertrophy.<sup>3</sup> Furthermore research with functional electrical stimulation of patients with long time denervated limb muscles showed very promising results.<sup>4</sup> The selective stimulation of denervated muscles has been investigated in rabbits with unilateral paresis of the recurrent laryngeal nerve. It could be shown that with triangular ramping and very long pulses (> 200ms) the afferent nerve fibers were not stimulated but only denervated muscle, with change in muscle fibers confirmed through histology.<sup>5,6</sup> It is to be investigated if these findings can be repeated with surface electrodes positioned in the neck area and successful stimulation of the denervated vocalis muscle can be performed without causing pain and excessive contraction of neighboring neck muscles rendering treatment impossible. The optimal stimulation parameters for this application and ideal position of the surface electrodes have yet to be investigated.

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### Determining optimal settings for selective surface stimulation in order to recruit paralyzed facial muscles under non painful conditions

Gerd Fabian Volk(1), Orlando Guntinas-Lichius(1), Tobias Schmid (1), Lukas Kneisz (2), Matthias Ladurner (2), Winfried Mayr (3)

(1) ENT Jena, Germany (2) MEDEL Innsbruck, Austria; (3) Center of Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria.

E-mail: [fabian.volk@med.uni-jena.de](mailto:fabian.volk@med.uni-jena.de)

Facial nerve paralysis as a peripheral nerve injury results in neuromuscular atrophy. The symptoms include significant aesthetic, functional and often life-altering consequences. Several procedures such as Nerve Grafting, Facial Reanimation and Rehabilitation have been developed to treat functional and cosmetic aspects of this disease.[1] Nerve grafting is a sophisticated surgery, which requires experience but offers promising results. Although cable grafting is state of the art, the method suffers the disadvantage of long nerve regrowth time. [2]Facial Pacing systems too show promising results to treat facial paralysis. [3] [4] Former research showed good results stimulating denervated extremity muscles using FES.[5] Nevertheless this field of research is still lacking optimal stimulation settings to selectively recruit denervated atrophic or simply age-related atrophic facial muscles under non painful conditions. Methods: Several Devices are considered to investigate optimal stimulation settings. To encourage noninvasive screening methods for facial pacing, surface electrodes are used to estimate the optimal settings for stimulations. The use of surface electrodes causes the need for optimized electrode positioning, which is also investigated. Results: Martin et al. [6] showed that recruitment of denervated muscles requires exponentially shaped pulses with long phase durations(>200ms). The outcome of our investigation confirms these findings as well, showing best performance when recruiting paralyzed facial human muscles with biphasic long-duration impulses. It is crucial to position the surface electrodes appropriately in order to avoid stimulation of innervated muscles, for instance the masseter muscle. Conclusions: Surface electrodes, combined with the optimal stimulation settings, offer a screening possibility for facial pacing. Since muscles affected by age-related atrophy could be recruited too, further research is necessary to show effectiveness of training using the determined exponential patterns.

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## **Vagus stimulation and other ES managements**

Jonathan C. Jarvis, Francesco Piccione, Chairs

### **Percutaneous Auricular Vagus Nerve Stimulation**

**Eugenijus Kaniusas (1), Stefan Kampusch (1), Jozsef Constantin Széles (2)**

(1) Institute of Electrodynamics, Microwave and Circuit Engineering, TU Wien, Austria; (2) University Clinic for Surgery, Medical University Vienna, Austria.

E-mail: [kaniusas@tuwien.ac.at](mailto:kaniusas@tuwien.ac.at)

Artificial stimulation of the vagus nerve, the main nerve of the parasympathetic nervous system, gained importance in the last years. Due to the modulatory interaction with the autonomous nervous system, the stimulation re-establishes the sympathovagal balance, counteracts over-inflammation responses or improves peripheral perfusion.<sup>1-3</sup> Thus, the clinical applications have a wide range from depression to acute/chronic pain or cardiovascular dysfunction up to various neurological disorders.<sup>3-7</sup> Neuromodulation is mediated via either implanted cervical, transcutaneous cervical/auricular or percutaneous auricular stimulation devices. While implanted stimulation devices are interrelated with high risks/costs and transcutaneous devices lack precision and require strong stimuli during their operation, percutaneous devices seem to avoid these drawbacks.<sup>8</sup> Current percutaneous stimulators use needle electrodes in the auricle to stimulate afferent nerve fibers by the use of simple monophasic or biphasic stimulation patterns with the need for an additional reference electrode.<sup>5</sup> No adaptation of the stimuli is possible to account for the specific pathology to be treated as well as the current physiological state of the patient.<sup>8</sup> Our group has developed a multi-punctual percutaneous stimulator which operates three independent stimulation channels without any additional reference electrode.<sup>8</sup> Stimulation patterns, with specific triphasic pulses, seem to reduce adaptation processes and to establish a pathology specific efficient stimulation. The pattern can be advantageously adapted throughout the stimulation duration to account for the current treatment state and physiological state of the patient [9]. Specific and precise positioning of needles - based on electrical and optical approaches<sup>10</sup> - close to auricular nerves is performed, which is of high importance for efficient nerve stimulation. Preliminary studies of our group show positive effects of this percutaneous stimulation on heart rate variability, cerebral/peripheral blood perfusion, pain, sleep, diabetic food syndrome and cervical dystonia.<sup>1,6,8,9,11,12</sup>

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## **"Noisy" electrical stimulation patterns facilitate the activity of cultured muscle cells**

**Marina Sciancalepore (1), Giuliano Taccola (2), Gaia Ziraldo (1), Tamara Coslovich (2), Paola Lorenzon (1)**

(1) Dept. Life Sciences and BRAIN Center, University of Trieste; (2) Dept. Neuroscience, SISSA, Trieste, Italy

E-mail: [msciancalepore@units.it](mailto:msciancalepore@units.it)

Use of electrical stimulation (ES) of skeletal muscle as a tool to restore normal control of movement and ability to perform motor tasks has lately received increasing attention. Its capability to elicit muscle tissue contractions through the delivery of current impulses is commonly exploited in clinical settings,<sup>1</sup> when damage to the nervous system, either central or peripheral, produces rapid denervation of muscle, resulting in weakness or paralysis. Possible mechanisms of muscle fibre recruitment have previously been studied using stereotyped electrical pulses delivered at variable pulse frequency, width and current amplitude.<sup>2</sup> However, these protocols often exhibit several significant limitations, resulting in an overall decreased efficiency of contraction, ultimately leading to the development of muscular fatigue, as well as the elicitation of unpleasant symptoms. In the present study, the influence that different parameters of ES protocols exert on the efficacy of skeletal muscle cell contractions was investigated in skeletal myotubes in



culture. The efficiency of a “noisy” stimulus waveform, derived from human muscle electromyogram (EMG) recordings, used as templates for the delivery of ES, was compared with conventional stereotyped 1 Hz and 40 Hz electrical stimulation delivery.<sup>3</sup> EMG traces obtained by recording human gastrocnemius medialis muscle activity during sessions of real overground locomotion, were used to design the “noisy” stimulation pattern (EMGstim). ES protocol efficiency in inducing contractile activity of cultured skeletal muscle cells was compared by measuring intracellular  $Ca^{2+}$  dynamics and patch-clamp electrophysiological recordings. Collected data demonstrated that EMGstim was more efficient in inducing myotube cell action potential firing,  $[Ca^{2+}]_i$  changes and contractions, when compared with more conventional electrical stimulation using stereotyped rectangular pulses. Furthermore, it was demonstrated that EMGstim strength was also considerably lower than the minimum current amplitude required to induce contractions via canonical stimulation protocols. These results demonstrate the peculiar properties of the “noisy” EMGstim pattern to enhance the efficiency of muscle cell recruitment, minimizing charge transfer and therefore preventing possible tissue damage. We suggest this could represent a promising new approach for the optimization of ES protocols and for future design of electrical devices to stimulate the rehabilitation/recovery of weakened or injured muscles in human patients.

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### Evaluation of tSCS treatment for the alleviation of lower limb spasticity

Halla Kristin Gudfinnsdottir (1), Jose Luis Vargas Luna (1,3), Vilborg Gudmundsdottir (2), Gigja Magnusdottir (2), Gudbjorg Kristin Ludvigsdottir (2), Thordur Helgason (1,2)

(1) Reykjavik University, Iceland; (2) Landspítali – University Hospital, Reykjavik, Iceland; (3) Tecnológico de Monterrey, Monterrey, Mexico

E-mail: [thordur@landspitali.is](mailto:thordur@landspitali.is)

Spinal cord injury is a traumatic injury of descending spinal cord tracts that alters the spinal neural circuitry.<sup>1-3</sup> Spasticity is a common result of spinal cord injury (SCI) and can restrict daily living activities, cause pain and fatigue and, therefore, decrease the quality of life for SCI individuals.<sup>4-5</sup> The aim of this study is to evaluate the effects of transcutaneous spinal cord stimulation (tSCS) on individuals with post-traumatic SCI for the alleviation of lower limb spasticity. Methods: In total, 8 subjects, 5 males and 3 females, aged between 31 – 63 years old ( $M = 49.9$ ;  $SD = 11.5$ ) were studied, with complete- and incomplete SCI. The evaluation of the effects of tSCS was done by means of electrophysiological evaluation and evaluation of residual motor control functions. The protocol consisted of four stages: first assessment/evaluation (control data), application of 30-min tSCS, a second assessment immediately after the treatment and a third assessment two hours after stimulation. The assessments consist of the evaluation of the spasticity level through the Ashworth

scale, clonus beet quantification, 10-m walking test (if possible), electrophysiological evaluation (Brain Motor Control Assessment, BMCA [1]) and the Wartenberg pendulum test (WPT).<sup>6</sup> Results: The index of spasticity R2n, derived from the WPT is the primary variable and the results of the WPT show increase in muscle tone in four subjects while the others presented average index values  $\geq 1$ , indicating non-spastic conditions. During the BMCA, there was a significant difference of the normalized EMG activity of all muscles before the stimulation and immediately after stimulation for all participants, which indicates amelioration of intrinsic phasic and extrinsic spasticity. Enhancement of motor control was also observed. Conclusion: The similarity of the effects of tSCS with those induced by epidural SCS, strongly suggests that both techniques are able to activate similar neural structures. From our results we can see that the application of low-intensity tSCS for 30 minutes leads to the alleviation of lower limb spasticity regardless of the clinical profile of the subjects and enhancement of voluntary motor control in the motor incomplete SCI subjects.

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### Quantifying muscle degeneration from nonlinear trimodal regression analysis of radiodensitometric CT distributions

Kyle J. Edmunds (1), Iris Árnadóttir (1), Magnus K. Gíslason (1), Ugo Carraro (2), Paolo Gargiulo (1,3)

(1) Institute for Biomedical and Neural Engineering, Reykjavík University, Reykjavík, Iceland; (2) IRCCS Fondazione Ospedale San Camillo, Venezia, Italy; (3) Department of Rehabilitation, Landspítali, Reykjavík, Iceland

E-mail: [kylejedmunds@gmail.com](mailto:kylejedmunds@gmail.com)

Whether via sarcopenia, cachexia, or sequela of trauma, the degeneration of muscle has been consistently identified as an independent risk factor for mortality.<sup>1</sup> Many recent investigations have realized the quantitative potential of CT image analysis to describe skeletal muscle volume and quality.<sup>2-4</sup> However, the optimum metric for assessing these data remains debated. Identifying a novel quantitative method for muscle assessment in this regard would allow for the generalizability of such studies to clinical practice and therefore aid in the indication of compensatory targets for clinical intervention. While there is much extant literature reporting the use of average HU values to investigate muscle quality and its utility as a comorbidity index, standardized methods for this analysis have yet to be defined, and no existing studies have explored the utility of an entire radiodensitometric distribution. Herein, we hypothesize that rigorously quantifying entire HU distributions can elicit much more information regarding muscle quality than extant methods that, to date, only utilize average HU



attenuation values. This study reports the development and use of this method, wherein we assess upper leg muscle quality utilizing nonlinear trimodal regression analysis with radiodensitometric distributions from computed tomography (CT) scans of a healthy young adult, a healthy elderly subject, and a spinal cord injury patient exhibiting complete lower motor neuron denervation. Results from this assessment highlight the utility of entire HU attenuation value distributions and identify novel parameters from these analyses that could provide further insight into how muscle degeneration can be optimally quantified.

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## Biomarkers in aging and neuromuscular disorders

S Masiero, U Carraro, Chairs

### Ca<sup>2+</sup>-handling biomarkers of skeletal muscle plasticity for neurorehabilitation

Simone Mosole (1,2,3,4), Sandra Zampieri (1,4), Angie Caon (1,2), Sandra Furlan (5), Hanna Fruhmant (4), Helmut Kern (4), Pompeo Volpe (1,2), Ugo Carraro (6), Alessandra Nori (1,2,4)

(1) Laboratory of Translational Myology of the Interdepartmental Research Center of Myology, Department of Biomedical Science, University of Padova; (2) Pathophysiology of Striated Muscles, Dept. Biomedical Sciences, University of Padova; (3) Plastic Surgery Clinics of the Department of Neurosciences, University of Padova, Italy; (4) Ludwig Boltzmann Institute of Electrical Stimulation and Physical Rehabilitation, Department of Physical Medicine and Rehabilitation, Wilhelminenspital Wien, Austria; (5) Neuroscience Institute of the Italian C.N.R., c/o Department of Biomedical Science, University of Padova, Italy; (6) IRCCS Fondazione Ospedale San Camillo, Venice, Italy

E-mail: [alessandra.nori@unipd.it](mailto:alessandra.nori@unipd.it)

Physical activity plays an important role in preventing muscle atrophy and chronic diseases in adults and the elderly. Voluntary physical exercise is not always feasible and other therapies should be applied such as electrical stimulation (ES).<sup>1</sup> The process of calcium storage, uptake and release (EC-coupling) and, in a broader framework, Ca<sup>2+</sup> cycling is essential in activity-induced muscle adaptation.<sup>2</sup> De-codification of Ca signals is accomplished by an heterogeneous class of decoders such as transcription factors (i. e NFATc1, PGC1α) kinases (CaMks) and phosphatases (Calcineurin).<sup>3,4</sup> We investigated the effects of either passive ES (acute or long-lasting) or voluntary physical exercise (leg press, LP), on expression of Ca<sup>2+</sup> handling proteins of the sarcoplasmic reticulum and on the activation of key Ca<sup>2+</sup> signal decoders in human vastus lateralis (VL) of elderly sedentary persons. Muscle sections and total homogenates were obtained from biopsies performed before and seven days after nine weeks of ES, before and 30 minutes after one session of ES and before and seven days after LP volitional exercise on a group of volunteers.<sup>1,5</sup> Expression of Sarcalumenin, SERCA and p-CaMKII were evaluated by western blot while NFATc1/PGC1α nuclear translocation and muscle remodeling were determined by immunofluorescence. Evidence of kinase and phosphatase activation after both ES and LP were obtained. NFATc1 translocation to nuclei 30 minutes after one ES session training was obtained, after 9 weeks of ES NFATc1 translocation lasted at least 7 days. Moreover, mixed SERCA 2/MHCII fibers and Ca<sup>2+</sup> handling proteins Sarcalumenin and SERCA 2 increased after ES. Conclusions. These results show that ES influences expression of muscle components deputed to Ca<sup>2+</sup> cycling and promotes fiber remodeling essential to improve muscle performance in old sedentary people. This work identifies a set of molecules which are modifiable by ES, easy to measure and gender and age independent suitable as biomarkers for skeletal muscle response to neurorehabilitation.

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### Biomarkers of skeletal muscle and bone regeneration-adaptation to neurorehabilitation training strategies

Barbara Zavan (1), Chiara Gardin (1), Letizia Ferroni (1), Simone Mosole (1), Ugo Carraro (2)

(1) Department of Biomedical Sciences, University of Padova, Padova; (2) IRCCS Fondazione Ospedale San Camillo, Venice, Italy

E-mail: [barbara.zavan@unipd.it](mailto:barbara.zavan@unipd.it)

Most organisms experience changes in regenerative abilities through their lifespan. The principles that underlie the decline in regenerative





abilities through lifespan are currently being unraveled. However, it is already clear that both cell-intrinsic (such as cellular senescence) as well as cell-extrinsic (such as alterations in the regenerative environment) factors play significant roles. During aging, numerous tissues exhibit a progressive decline in homeostasis and regeneration that results in tissue malfunction, pathology and degeneration. With age, both stem and progenitor cells undergo a series of alterations including loss of self-renewal capacities, altered proliferative activity, declines in functionality and potency. These changes have been shown to contribute to the dysfunction and degeneration of a number of tissues and systems including most epithelia and endothelia, blood, skeletal and cardiac muscle, bone, cartilage, the peripheral and central nervous system (CNS), and organs such as the pancreas, liver, kidney and lungs. The regenerative capacity in the skeletal muscle system experiences a marked decline with age in many organisms, as reflected by a decrease in the generation of muscle fibres and an increase in fibrotic tissue upon muscle injury. In humans, this is an underlying cause of sarcopenia, the loss of muscle mass that accompanies late aging. The decline in muscle regenerative potential is largely attributed to changes in satellite cells, the muscle stem cells, which undergo age-related declines in proliferative and myogenic capacities. Indeed, satellite cell numbers decline gradually in mammalian muscles with advancing age. Age-specific changes have also been reported for mesenchymal stem cells (MSCs), stromal cells that can differentiate into multiple cell types such as osteoblasts, chondrocytes, and adipocytes. Alterations include a loss in chondrogenic potential leading to impaired chondrocyte formation, which results in decreased cartilage repair in aged mammals. Furthermore, studies in human-derived bone marrow MSCs revealed age-dependent decreases in their capacity to differentiate to osteoblasts, which are related to increases in the level of MSCs senescence and apoptosis upon aging. Together, these alterations contribute to conditions such as osteoporosis and reduced bone repair capacity that are characteristic of human aging. A number of cellular and molecular mechanisms have been associated with the decline in regenerative abilities observed during aging in humans. These include intrinsic factors such as genomic instability (including telomere attrition), mitochondrial dysfunction, epigenetic changes, loss of proteostasis and metabolic alterations, as well as cell-extrinsic factors such as disruption of the regeneration niche and alterations in systemic signals. Though most of these factors can contribute to age-related impairment in regenerative capacity, a consensus on their relative importance in this process is currently lacking. Furthermore, emerging evidence suggests a high degree of interconnectivity between them, stressing the importance

of identifying the common denominators. The advances in our understanding of the factors that modulate the decline in regenerative abilities have pinpointed areas of potential clinical relevance. In this view examining the influence of systemic factors on aged progenitor cells from tissues activated during neurorehabilitation training may prove to be clinically relevant. Our activity will be focused on the study of 3 different markers present in blood and tissue biopsies, such as long and small noncoding RNAs,<sup>1-3</sup> growth factors,<sup>4-6</sup> and transcription factors, such as NFAT.<sup>7,8</sup>

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