

## Report and Abstracts of the 14<sup>th</sup> Meeting of IIM, the Interuniversity Institute of Myology, - Assisi (Italy), October 12-15, 2017

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### Abstract

The 14<sup>th</sup> Meeting of the Interuniversity Institute of Myology (IIM), October 12-15, 2017 - Assisi, Italy gathered together researchers from Italy, European and North-American countries to discuss recent results on muscle research. The program showcased keynote lectures from world-renowned international speakers presenting advances in muscle physiology, bioengineering, metabolism and therapeutics. Based on selection from submitted abstracts, participants presented their novel, unpublished results in seven oral communication and two poster sessions. Particular emphasis was devoted to young trainees. For example, trainees were directly involved in organizing a scientific session and three round tables tailored to the interests of their peers. The meeting attracted a broad audience from Italy, various European countries and from North America. It offered a unique opportunity to all researchers involved in the field of muscle biology to exchange ideas and foster scientific collaborations to better understand the causative mechanisms of muscular diseases and to improve the design of more efficient therapeutic strategies. The friendly and inclusive atmosphere promoted the active participation of junior scientists to exciting discussions, which allowed to identify emerging areas of myology research and encouraged scientific cross-fertilization to facilitate exchanges between different laboratories in different countries. The meeting was a success and this community will continue to deliver major contributions to our understanding of muscle development and function, the pathogenesis of muscle diseases and the development of novel therapeutic approaches. Here, we report abstracts of the meeting discussing recent results of basic, translational and early clinical studies confirming that the field of Myology is strong and articulated, maturing toward clinical development for the treatment of muscle diseases.

**Key Words:** muscle development, stem cells, regeneration, epigenetics, muscle wasting, proof of concept, translational myology, *ex-vivo* and *in-vivo* studies, clinical trials

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Founded in 2003, the Interuniversity Institute of Myology (IIM) brings together investigators performing muscle research in Universities and Research Centers in Italy and abroad. IIM mission is to promote interdisciplinary collaborative efforts to further the understanding of normal and pathological aspects of muscle formation, activity and repair, as well as to foster collaborations between researchers, clinicians and patient associations for the development of therapeutic treatments for neuromuscular diseases. Special emphasis is devoted to the training of young fellows, and to promote international collaborations and exchanges.

At the recent 14<sup>th</sup> IIM Meeting, held in Assisi, Italy October 12-15, 2017, junior and senior myologists from Italian Universities and Research Centers gathered with international colleagues to present recent biomedical and clinical results. Forty four Oral Communications were grouped in seven Sessions: 1. Signaling in muscle growth, homeostasis and disease; 2. Biophysics and E-C coupling in the pathophysiology of neuromuscular diseases; 3. Satellite cells and muscle regeneration in healthy muscle and in diseases; 4. Genetic and epigenetic alterations in muscle dystrophies and myopathies; 5. Metabolic alterations and muscle diseases; 6. Therapeutic approaches for muscle diseases;

7. Muscle wasting, sarcopenia and cachexia. Thirty five Poster presentations were always on display during the entire Meeting. Four Keynote Lectures of Marco Linari (PhysioLab, University of Florence, Italy), Nicola Elvassore (Venetian Institute of Molecular Medicine, Padova, Italy), Jorge Ruas (Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden), and Mark Tarnopolsky (McMaster University Medical Center, Hamilton, ON, Canada) completed the Program of the three days of the Meeting. Scientific discussions were very interesting and new collaborations were developed during the scientific sessions and through spontaneous gatherings of several after lunch and dinner. The international participation is increasing every year, a strong evidence that the Italian community of myologists has high standards and is able to attract young and senior scientists.

The meeting has been highly successful and instrumental to facilitate exchanges between different laboratories of different countries. It was also very valuable for identifying emerging areas of research and to encourage the active participation of qualified researchers and junior scientists in the field. In particular, the IIM-Myology meeting offered an unique opportunity to all researchers involved in the field of myology to exchange ideas and stimulate scientific collaborations in order to better understand the causative mechanisms of muscular diseases and to improve the design of more efficient therapeutic strategies. It is very likely that this community will continue to deliver major contributions to the understanding and treatment of muscular muscle diseases. Moreover, there is an urgent need to integrate all the themes involved in myology research in a setting that promotes discussion. This will certainly attract a broader audience and contribute to scientific cross-fertilization in this exciting area of research.

On the first evening of the meeting, young scientists organized three Roundtable Discussions during which trainees discussed about issues and new ideas concerning Gene therapy, stem cells e bioengineering; Metabolism, exercise and signaling pathways in muscle wasting; Muscular dystrophies. Moderated by the Invited Speakers or selected members of the IIM Scientific Committee, the roundtables offered an ideal opportunity to discuss muscle research in an informal way, over food and drinks provided by local producers. On the second evening of the meeting, guided tours were organized to the Saint Francis Basilica famous for its Giotto's frescoes and to the ancient Roman forum. The evening concluded with a medieval show and dinner in the historic *Sala delle Volte*. Finally, an Award Ceremony was held on the last evening of the meeting to congratulate Martina Baraldo, Ester Sara di Filippo, Anais Franco Romero and Alessandra Pasut, winners of prizes for best Oral and Poster presentations selected by an international panel composed by the Invited Speakers and members of the IIM Scientific Committee.

Since 2014, the collections of abstracts presented to the annual IIM Meeting are e-published in the European Journal of Translational Myology, an Open Source Journal whose contents are retrievable in PUBMED from 2014 and more recently in the Web of Science. The collections of abstracts provide information on research lines active on basic and translational myology.<sup>1-4</sup> In EJTM more clinically oriented myology research activities, related to muscle repair and rehabilitation, are published in the collections of abstracts of the PaduaMuscleDays, a series of Meetings that held twice a year in Euganei Hills and Padua, Italy.<sup>5-9</sup> EJTM is also publishing articles and reviews submitted by researchers of the Italian Community of Myologists.<sup>10-30</sup>

Here,<sup>4</sup> the abstracts of the 14<sup>th</sup> IIM Meeting show that results of basic, translation and early clinical studies for many topics are mature to be translated to clinical application. However, further studies will be necessary to validate clinical relevance of these preliminary results. Hopefully, public and private granting agencies will provide the funds to support young and expert researchers needed to implement sound clinical trials.

#### Author's contributions

Authors equally contributed design, write and approved the proofs of the manuscript.

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#### Conflict of Interest

None of the authors have conflicts of interests.

#### Ethical Publication Statement

Authors confirm that they have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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## Abstracts of the 14<sup>th</sup> IIM Meeting, Assisi, Italy - October 12-15, 2017.

### LECTURE 1

#### Breaking news on the regulation of striated muscle contraction: thick filament participates!

**Marco Linari**

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Force and shortening in striated (skeletal and cardiac) muscles are generated at the level of the sarcomere, the structural unit, by the two bipolar arrays of the motor protein myosin II emerging from the thick filament, during cyclical interactions with the interdigitating thin, actin-containing, filaments. In the classical model of striated muscle regulation the OFF (resting) and ON (actively contracting) states of muscle correspond to the OFF and ON state of the thin filament and the switch between states is controlled by calcium. However, in the resting muscle most of the myosin motors lie on the surface of the thick filament folded back against the myosin tails unavailable for binding to the actin (Woodhead et al. *Nature* 436:1195, 2005; Zoghbi et al. *PNAS* 105:2386, 2008) and splitting ATP (Stewart et al. *PNAS* 107:430, 2011) (OFF state of the thick filament). This implies the presence of a second thick-filament-based switch for contraction: muscle is ON when both the thin and thick filaments are ON. To investigate this point, we used sarcomere-level mechanics and X-ray diffraction interference from striated muscle cells at ID02 beamline of the European Synchrotron (Grenoble, France). In single intact fibres from frog skeletal muscle we showed that, for contraction against high loads, the recruitment of myosin motors from the OFF state is based on a stress-sensing mechanism in the thick filament and that during unloaded shortening a large fraction of motors recovers progressively the OFF state (Linari et al. *Nature*, 528:276, 2015). The role of thick filament stress-sensing in heartbeat regulation has been

investigated by exploiting our recent development of sarcomere-level mechanics in intact trabeculae isolated from the ventricle of rat heart (Caremani et al. *PNAS* 113:3675, 2016) and the possibility at ID02 beamline to record both the nanometer-scale signals from the contractile proteins along the thick filament (M1-M6, camera length 1.6 m) and the micrometer-scale changes of the length of the sarcomere (camera length 30 m). It was found that, starting from the same resting sarcomere length, at the peak of the twitch force the intensity profile of the M3 reflection due to axial repeat of myosin motors change with the loading conditions, whether sarcomere shortening against the end compliance during force development is allowed (fixed end conditions) or is prevented (length clamp conditions) (Reconditi et al. *PNAS* 114:3240, 2017). The difference in M3 intensity profiles indicates that, during a cardiac twitch, only a fraction of the motors leaves the OFF state and this fraction depends on the level of the force independently of the diastolic sarcomere length. The different loading conditions of the twitch reproduce the conditions, at the organ level, of the left ventricle beating against a high or a low aortic pressure. We conclude that a stress-sensing mechanism in the heart tunes the energetic cost of contraction to the mechanical task.

### LECTURE 2

#### The role of endogenous signals in cellular reprogramming and programming

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**LECTURE 3****Transcriptional regulation of skeletal muscle function: local and systemic impact**

Jorge Ruas

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Skeletal muscle adapts to exercise training through the concerted actions of several transcriptional regulators, among which PGC-1 $\alpha$  coactivators play a central role. When activated by aerobic exercise, PGC-1 $\alpha$  induces genes relevant to mitochondrial biogenesis, adaptive thermogenesis, lipid and glucose homeostasis, fiber-type switching, among other processes. In addition, exercised skeletal muscle promotes peripheral kynurenine detoxification, which protects from stress-induced depression. Metabolites of the kynurenine pathway of tryptophan degradation play important roles in the regulation of neuroinflammation and mental health. This process is mediated by PGC-1 $\alpha$ , which enhances kynurenine aminotransferase (KAT) gene expression in skeletal muscle. Elevated KAT levels convert accumulating kynurenine into kynurenic acid, an end metabolite of the pathway that cannot cross the blood-brain barrier and therefore remains in the periphery. We will discuss some of the consequences of activating muscle kynurenine metabolism for peripheral tissue inflammation and energy expenditure.

**LECTURE 4****Effect of exercise on cellular senescence and systemic mitochondrial function**

Mark Tarnopolsky

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Human aging is associated with an increase in the number of senescent cells in a variety of tissues. Cellular senescence has been described in vitro as the “Hayflick phenomenon”; whereby, cells fail to replicate. Cellular senescence is associated with other canonical features of aging such as telomere shortening, an increase in free radicals/reactive oxygen species, mitochondrial dysfunction and an increase in inflammatory markers. The senescence associated secretory pattern (SASP) refers to the propensity for senescence cells to release pro-inflammatory cytokines (IL-1  $\alpha$ , IL-1  $\beta$ , IL-6, IL-13), proteases (PAI-1, MMP-3, MMP-1, TIMP-1), growth factors (hepatocyte growth factor, basic fibroblast growth factor, VEGF- $\alpha$ , HGF, EGF) and chemokines (IL-8, CXCL, eotaxin, eotaxin-3, MCP2, IL-8, MIP1A). We and others have found that the basal concentration of many of the constituents of the SASP are higher in older humans and mice and are higher in progeroid aging (polymerase  $\gamma$ -1 mutator mouse model). Our recent data shows that fibroblasts from older adults have far fewer replicate cycles than those derived from

younger adults and older athletes are similar to the young sedentary adults. Furthermore, fibroblasts taken from sedentary older adults have greater number of passages until cellular senescence following three months of endurance exercise training. We and others have shown improvements in mitochondrial function and lower oxidative stress in both cross sectional and longitudinal studies in humans and mice. Acute endurance exercise often leads to a “pulse” of higher SASP components; however, long term endurance training leads to a lower basal level of SASP. Given that the consistent observation of increased mitochondrial function and lower oxidative stress in endurance trained mice and humans and the importance of mitochondria to senescence pathways (telomere length, oxidative stress, inflammasome activation) I posit that mitochondrial function is the main factor influenced by exercise training that leads to a reduction in SASP and fewer senescence cells.

			October 12 <sup>th</sup>
<b>SESSION 1.</b>			
<b>SIGNALING</b>	<b>IN MUSCLE</b>	<b>GROWTH,</b>	
<b>HOMEOSTASIS</b>	<b>AND DISEASES</b>		

**1.1 SH3BP2 as a novel scaffold protein regulating muscle postsynaptic machinery**

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Neuromuscular junctions (NMJs) are synapses formed between motor neurons and skeletal muscle fibers. Abnormalities in NMJ development lead to various neuromuscular disorders, which are often fatal. Despite their crucial role, the mechanisms that orchestrate NMJ development are still poorly understood. The Dystrophin-associated Glycoprotein Complex (DGC) is a major laminin receptor in the muscle required for proper development of the postsynaptic machinery by linking its components to the extracellular matrix and the actin cytoskeleton. One of the cytoplasmic DGC-associated proteins,  $\alpha$ -dystrobrevin-1 ( $\alpha$ DB1), was shown to play an important role in the organization of the NMJ postsynaptic machinery. For its proper functioning  $\alpha$ DB1 needs to be phosphorylated on its C-terminal fragment. To gain insight into the molecular mechanism of  $\alpha$ DB1 function we have recently performed a biochemical screen for phospho-specific interacting proteins and identified SH3BP2 as a binding partner. SH3BP2 is a scaffold protein with unknown localization and function in skeletal muscles. We demonstrate that SH3BP2 is concentrated at the NMJ postsynaptic machinery and also at the muscle

contractile machinery. Cultured myotubes depleted of SH3BP2 had the impaired ability to cluster AChRs. A similar phenotype was observed at the NMJ upon muscles-specific deletion of SH3BP2. Protein complex purification experiments combined with mass spectrometry analysis revealed that SH3BP2 interacts with several postsynaptic proteins including Lrp4, AChR, and CK2, proteins of the muscle contraction machinery as well as several components of the DGC. Our results suggest that SH3BP2 acts as a scaffold protein involved in the organization of the NMJ postsynaptic specialization.

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### 1.2 Identification of a novel FoxO-dependent regulator of muscle mass

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Muscle atrophy results from transcriptional adaptations occurring in catabolic conditions. The Forkhead Box (Fox) transcription factors FoxO1, FoxO3 and FoxO4 are critical mediators of the catabolic response in skeletal muscle. An exacerbated activation of FoxO family members leads to increased protein breakdown and muscle wasting. Microarray analysis during fasting showed that FoxOs are required for the induction of several atrophy-related genes (atrogenes). However, the activation of already identified atrogenes cannot sustain all the protein breakdown during atrophy. Indeed, the discovery of new unknown players involved in muscle protein degradation is now of potential interest. We identified several new FoxOs-dependent genes, called Rikens, whose functions are still unrevealed. We showed that some Rikens are up-regulated in catabolic condition such as fasting, disuse and cancer cachexia. Interestingly, knocking-down one of the Rikens (Riken1) protected from atrophy during fasting. Moreover, colocalization experiments showed a possible Riken1-LC3/Riken1-Lamp1 interaction, suggesting a potential role in the autophagy-lysosome pathway. Our findings will contribute to the identification of new mediators of muscle mass loss in order to develop new therapeutic approaches against muscle wasting.

### 1.3 The physiopathological role of mitochondrial calcium uptake in skeletal muscle homeostasis

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Muscle activity leads to major swings in mitochondrial  $[Ca^{2+}]$ , which control aerobic metabolism, survival pathways and cell death. Recently, we showed that mitochondrial  $Ca^{2+}$  uptake positively modulates skeletal muscle trophism by impinging on two major pathways, PGC-1 $\alpha$ 4 and IGF1-AKT/PKB thanks to the use of AAV vectors. Here, we aimed to discern the metabolic route regulated by mitochondrial  $Ca^{2+}$  uptake that is responsible for muscle trophism. For this purpose, we generated a skeletal muscle specific Mcu knockout mouse (*mlc1f-Cre-Mcu<sup>-/-</sup>*), by crossing a *Mcu<sup>fl/fl</sup>* mouse with a line expressing the Cre recombinase under the control of the myosin light chain 1f (*mlc1f*) promoter. Our preliminary data confirm that PGC-1 $\alpha$ 4 and IGF1-AKT/PKB signaling pathways are negatively regulated in skeletal muscle specific *Mcu* knockout animals. In addition, we also observed a slight decrease of fibre size in *mlc1f-Cre-Mcu<sup>-/-</sup>* skeletal muscles. Most importantly, when these mice were exercised on a treadmill using different training protocols, an impaired running capacity became evident, indicating that mitochondrial  $Ca^{2+}$  accumulation is required to guarantee skeletal muscle performance. Finally, a clear metabolic alteration is present in *mlc1f-Cre-Mcu<sup>-/-</sup>* animals. Specifically, *mlc1f-Cre-Mcu<sup>-/-</sup>* mice show decreased glucose, increased lactate, free fatty acids and ketone bodies, suggesting an impaired crosstalk between skeletal muscle and liver. Taken together, these data indicate that mitochondrial  $Ca^{2+}$  uptake plays a pivotal role in the control of skeletal muscle trophism. Further investigations of MCU-dependent effects on skeletal muscle homeostasis will represent an important task for the future. Indeed, this research will provide new possible targets for clinical intervention in all diseases characterized by muscle loss, such as dystrophies, cancer cachexia and aging.

### 1.4 Mitochondria and Cytoskeleton rearrangement in Drp1 overexpressing skeletal muscle

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In skeletal muscle mitochondrial fusion and fission define the mitochondrial network morphology regulating myofiber differentiation, muscle contraction and response to stress conditions. Mitochondrial fission is mainly mediated by dynamin-related protein 1 (Drp1) which represents a critical player in myogenesis and its inhibition suppresses myotube formation. We studied a transgenic mouse overexpressing Drp1 specifically in skeletal muscle (Drp/MC). We have already showed growth defects of Drp/MC mice starting from P7 mainly due to an impairment of glycolytic muscles development; they display an overall 20% reduction of body weight at P100 and a drop of locomotor performance without any increasing in catabolic event. Drp/MC mice exhibit low mitochondrial DNA levels which trigger mitochondrial stress and upregulate the unfolding proteins response (mtUPR) together with an impairment of Growth Hormone anabolic pathway. Interestingly we observe a strong remodeling of mitochondria distribution with a depletion of inter-myofibrillar mitochondria and an enrichment of the sub-sarcolemma pool. In parallel, we observe a perturbation of cytoskeleton framework characterized by the disruption of Desmin network (the main skeletal muscle intermediate filament connecting mitochondria to cytoskeleton) with the presence of Desmin aggregates inside myofibers and accumulation beneath the sarcolemma. Our study aims at identify the involvement of different molecular effectors, such as the kinesin Kif5b and myotubularin, in dysregulated muscular and mitochondrial phenotype of Drp/MC mice.

### 1.5 Unravelling MYO9A pathophysiology in CMS

**Emily O'Connor** (a), Vietxuan Phan (b), Isabell Cordts (a), George Cairns (a) Andreas Roos (a,b), Hanns Lochmüller (a)

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Congenital myasthenic syndromes (CMS) are a group of rare, inherited disorders characterised by compromised function of the neuromuscular junction (NMJ) manifesting with fatigable muscle weakness. We identified mutations in MYO9A as causative for CMS but the precise pathomechanism remained to be characterised. We hypothesised that defects in MYO9A affect the neuronal cytoskeleton, thus leading to impaired vesicular transport. MYO9A-depleted NSC-34 cells (mouse motor neuron-derived cells) were used to assess the effect on the cytoskeleton using immunofluorescent and immunoblotting techniques. Vesicular transport was analysed using different assays including a secretomic study to identify factors released from the nerve to act on the muscle fibre for NMJ development and function. In addition, an unbiased approach utilising proteomic profiling of control and

MYO9A-depleted NSC-34 cells was performed to identify key players of the pathophysiology. Disruption of the cytoskeleton has been identified in MYO9A-depleted cells, with corresponding defects in receptor recycling and regular transport of proteins to the cell surface also observed. Proteomic data support a role for defective vesicular transport and identified affected proteins which are also involved in the manifestation of other neuromuscular disorders. Furthermore, a therapeutic target was identified and treatment of our MYO9A zebrafish model was able to ameliorate movement defects, increase muscle mass and improve muscle innervation. Our combined data allow new insights into the pathophysiology of CMS and show that loss of MYO9A affects the neuronal cytoskeleton, leading to impaired transport and vesicular recycling of proteins. We also identified a protein of potential therapeutic importance for patients.

### 1.6 Organelle pathology affecting protein processing and clearance in Myotonic Dystrophy type 1

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Myotonic Dystrophy is the most common adult onset muscular dystrophy and 2 major forms have been identified based on distinct genetic mutations. Myotonic Dystrophy Type 1 (DM1) is caused by (CTG)<sub>n</sub> trinucleotide repeat expansion in the 3' untranslated region of the DMPK gene and displays an autosomal dominant mode of inheritance. Patients present with myotonia, muscular weakness, cardiac arrhythmia, and visual disturbances. The repeat expansion causes the disease through a toxic RNA product clustering RNA processing factors. Mis-splicing of diverse transcripts has been observed in DM1 resulting in presence of aberrant proteins and/or perturbed protein abundances such as for the SR resident SERCA. Affection of the latter protein leading to perturbed Ca<sup>2+</sup> SR homeostasis is in accordance with the few descriptions of perturbed ER/SR-homeostasis. As the ER/SR is connected to the Golgi, an affection of these protein processing compartments is very likely and presumably affects folding and glycosylation of further proteins. However, as there remains a lack in the understanding of organelle pathology in DM1, we here systematically addressed this topic: utilizing patient and animal model-derived material we studied (i) organelle integrity, (ii) cellular

fitness, and (iii) protein abundances (proteomics and immunoblot) including (iv) the study of glycoproteins. Moreover, (v) RNA-Seq and (vi) measurements of protein clearance have been performed. Our combined data reveal profound overall affection of the ER-Golgi machinery along with affection of the protein clearance machinery and thus strengthen the current understanding of the etiology of DM1 and hereby open new avenues for therapeutic intervention concepts.

### 1.7 Lacking of Nfix in macrophages induces a defect of muscle regeneration through failed phenotypical switch

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Muscle development and skeletal muscle regeneration are processes which required specific and synchronized controlled steps. In the laboratory, it has been shown that the transcription factor Nfix is necessary for both processes. During muscle development, Nfix is expressed by fetal myoblasts and regulates the switch from embryonic to fetal myogenesis by activating or repressing specific myogenic genes. In post-natal life, Nfix is expressed by satellite cells (SCs) regulating the proper timing of muscle regeneration upon injury. Interestingly, not only SCs but also macrophages (MPs) express Nfix. Nfix is expressed by MPs at the later stages of muscle regeneration. Specifically, Ly6C<sup>+</sup> pro-inflammatory MPs exhibit the same level of Nfix while the percentage of Nfix<sup>+</sup> Ly6C<sup>-</sup> anti-inflammatory MPs always increases over the time during regeneration. Since SCs also express Nfix, we generated LysMCre:Nfix<sup>fl/fl</sup> mice which selectively lack of Nfix in MPs. After cardiotoxin injection, skeletal muscles of LysMCre:Nfix<sup>fl/fl</sup> mice exhibit a delay of regeneration characterized by a persistence of necrotic myofibers and a later appearance of newly-formed myofibers. In vitro, Nfix silencing leads to a defect of anti-inflammatory phenotype acquisition and it maintains a pro-inflammatory phenotype upon M2 polarization. Functional experiments on myoblasts shown that LysMCre:Nfix<sup>fl/fl</sup> M2 MPs adopt WT M1 features. Moreover, we observed that MPs failed to switch from pro- to anti-inflammatory phenotype in vivo. Dystrophies are genetic muscle diseases characterized by continuous cycles of regeneration-degeneration, leading to exhaustion of SCs pool and inflammation. The high amount of MPs infiltration is linked to fibrosis establishment. Interestingly, we observed in two models of dystrophic mice (Sgca null and mdx) that the number of MPs positive for Nfix increases with the progression of the disease. With these studies, we are identifying Nfix as a necessary new interplayer for MPs identity and function during acute muscle regenerative process,

while Nfix seems deleterious in chronic dystrophic context.

### 1.8 Signaling pathways controlling Nfix expression in prenatal and postnatal skeletal muscle progenitors

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The transition from embryonic to fetal myogenesis is a crucial switch, required for the complete maturation of skeletal muscle. The transcription factor Nfix, specifically expressed during fetal myogenesis, is the master regulator of this transition. Here, we show that the temporal progression of prenatal muscle development is timed by the RhoA/ROCK axis, which maintains the embryonic myogenesis, suppressing Nfix expression. RhoA and ROCK elicit their effects repressing ERK kinase activity, which instead promotes the fetal genetic program. Thus, RhoA/ROCK/ERK axis constitutes one of the major pathways that regulate the temporal progression of prenatal muscle development through the control of Nfix expression. RhoA/ROCK and ERK signaling pathways were also active in the Satellite cells, the postnatal population of myogenic stem cells, which expresses high levels of Nfix. The modulation of RhoA and ROCK axis shows that their role, as Nfix inhibitors, is not conserved in Satellite cells. Conversely, ERK kinases activity is necessary for the induction of Nfix expression not only in prenatal fetal myoblasts but also in Satellite cells. In the light of the fact that dystrophic muscles lacking Nfix have a robust recovery of symptoms and muscle morphology, our results build the basis of a future therapeutic approach for muscular dystrophies by using ERK inhibitors, which are drugs currently used in clinic for the treatment of melanoma.

**October 12<sup>th</sup>**  
**SESSION 2**  
**BIOPHYSICS AND E-C COUPLING IN THE PATHOPHYSIOLOGY OF NEUROMUSCULAR DISEASES**

### 2.1 Mechanisms underlying aerobic training as a strategy to prevent episodes related to heat stress

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Heat stress is defined as perceived discomfort and physiological strain associated with exposure to a hot environment especially during physical work. In 2009, Dainese and colleagues demonstrated that mice lacking calsequestrin 1 knockout (CASQ1-null) suffer lethal episodes when exposed to high environmental heat (i.e. heat strokes) (Dainese et al. 2009). More recently, a link between hyperthermic episodes and excessive oxidative stress has been established (Michelucci et al. 2015). Finally, we discovered that crisis can be prevented by aerobic training, and that oxidative stress is decreased in muscles of mice that survive from heat exposure after training (Guarnier et al. IIM 2016). However, the mechanisms underlying the protective effect of aerobic training deserve additional investigation. Here, C57Bl/6 and CASQ1-null male mice had their individual maximal exercise capacity evaluated at 2-2.5 months of age before being subjected to aerobic training for 2 months (60% of maximal speed, 5x/week). In addition, groups of C57Bl/6 and CASQ1-null with the same age were sacrificed and muscles collected to evaluate mitochondrial damage and proteolysis, and cytochrome c and SERCA activities. At 4-4.5 months of age, controls, untrained and trained mice were first re-evaluated: CASQ1-null displayed a raised aerobic capacity and, when submitted to the heat stress protocol (41°C/1h), the mortality rate of trained CASQ1-null was greatly reduced (16.6%) when compared to untrained mice (85.6%). Muscles from CASQ1-null mice that survived from heat-stress protocol revealed that aerobic training was effective in: a) decreasing mitochondrial damage (13% vs 7 % in trained mice); b) improving energy generation, by increasing cytochrome c activity, while decreasing SERCA's; and finally c) decreasing calcium-dependent proteolytic activity. Taken together, these results indicate that the protective effect of aerobic training against heat strokes is mediated by reduction of oxidative stress which, in turn, reduces mitochondrial damage and proteolysis and improves energy expenditure.

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## 2.2 Exercise prevents formation of Tubular Aggregates in ageing skeletal muscle fibers

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Tubular aggregates (TAs), ordered arrays of sarcoplasmic reticulum (SR) tubes, are frequently found in ageing fast-twitch fibers of male C57Bl6 mice. TAs are also found in biopsies from patients affected by

Tubular Aggregate Myopathy (TAM), a muscle disorder linked to mutations in *STIM1* and *Orai1*, *STIM1* and *Orai1* are the two main players in store-operated  $Ca^{2+}$ -entry (SOCE), a mechanism that allows recovery of extracellular  $Ca^{2+}$  during repetitive activity and fatigue. First, we investigated presence of TAs, expression and subcellular localization of *STIM1* and *Orai1*, and contractile force during repetitive stimulation in EDL muscle from male adult (4 months) and aged (24 months) mice. While TAs are never found in 4 month old muscle, 50 % of fibers contains TAs in aged EDLs. Furthermore, we have found that: i) ageing causes *STIM1* and *Orai1*, to accumulate in TAs; and ii) EDL muscles from aged mice exhibit a decreased capability to maintain contractile force compared to young animals (relative force after 10 tetani:  $55.0 \pm 1.9\%$  vs.  $65.6 \pm 3.5\%$ ). Secondly, we analyzed EDL muscles from 3 male mice that were exercised in wheel cages for voluntary running for 15 months, from 9 up to 24 months of age (each mouse ran on the average a total of  $\sim 396 \pm 29$  km) and discovered that: a) wheel cage running significantly reduced formation of TAs, with a great reduction in the percentage of fibers containing TAs (50% vs. 7%, respectively in untrained vs. trained animals); b) EDL muscles from aged mice trained in wheel cages exhibit an increased capability to maintain contractile force compared to untrained aged match animals (relative force after 10 tetani:  $63.1 \pm 4.9\%$  vs.  $55.0 \pm 1.9\%$ ). Taken together these findings point to: a) TAs as structures that lead to dysfunctional accumulation of *STIM1* and *Orai1* in inactive mice; and b) voluntary exercise as an effective measure to prevent formation of TAs and maintain a functional SOCE in muscle.

## 2.3 New Biomarkers in Amyotrophic Lateral Sclerosis Etiopathogenesis: Analysis of the Biophysical Properties and Gene Expression in Skeletal Muscle of SOD-1 Mouse Models and Repurposing of Acetazolamide as a New Therapeutic Approach

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Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by motor-neuron injury and skeletal muscle atrophy. Transgenic animals carrying mutations in the SOD-1 gene develop similar symptoms than those observed in clinic. In this animal model skeletal muscle has been demonstrated to

be primarily involved in SOD-1-mediated toxicity (Dobrowolny et al. Cell Metabolism. 8, 425–36, 2008). In this context, sarcolemma ion channels play a crucial role for muscle function. Resting chloride conductance (gCl), sustained by the CIC-1 channel, controls sarcolemma excitability, indeed a large reduction of gCl produces myotonic-like symptoms (Pierno et al. Brain 125, 1510–21, 2002). At the moment, there are no data describing the involvement of skeletal muscle ion channels functions in this pathology, thus, we measured the resting gCl and the potassium conductance (gK), as well as muscle excitability in extensor digitorum longus muscle of 4-months old transgenic SOD-1 mice, at the onset of the symptoms, by using the two-intracellular microelectrodes technique. We found that resting gCl was strongly reduced in SOD-1 mice as compared to wild-type (WT), being it  $1593 \pm 100 \mu\text{S}/\text{cm}^2$  (19 fibers) and  $2410 \pm 79 \mu\text{S}/\text{cm}^2$  (22 fibers), respectively. Resting gK was increased in SOD-1 animals by  $67 \pm 27\%$ . Preliminary patch clamp studies showed different activity of the KATP channels and an altered sensitivity to ATP in accord with the increase of gK. Also sarcolemma excitability, evaluated as the maximum number of action potentials, was accordingly increased from  $7.1 \pm 0.8$  (16 fibers) in WT to  $12.6 \pm 1.5$  (10 fibers) in SOD-1 muscle fibers. Resting intracellular calcium level was increased in these animals and an altered response to caffeine was found. In order to evaluate the muscular involvement in the pathology we also examined an animal model in which the mutated SOD1 G93A gene is selectively overexpressed in skeletal muscle under the control of the MLC promoter. Similar modifications were found in skeletal muscle of these animals, since resting gCl was reduced to  $1694 \pm 146 \mu\text{S}/\text{cm}^2$  (19 fibers). We observed that the CIC-1 mRNA expression was significantly decreased in muscles of SOD-1 mice respect to WT but do not change in muscles of MLC/SOD1G93A mice. In this animal model we tested the in vitro effect of acetazolamide, previously found to beneficially improve CIC-1 function in Myotonia Congenita through voltage-dependence regulation (Desaphy et al. Exp Neurol. 248: 530-40, 2013). Interestingly, acetazolamide application, increased resting gCl toward the control value, being  $2097 \pm 119 \mu\text{S}/\text{cm}^2$  (19 fibers). Accordingly, sarcolemma hyperexcitability was improved. In conclusion, chloride channel function is modified in skeletal muscle of the SOD-1 transgenic animals suggesting their contribution to ALS and opens the possibility to investigate on acetazolamide as a promising therapy.

#### 2.4 Physiological and structural properties of the Octopus vulgaris arm hydrostatic muscles

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The octopus arm is composed by uninucleated myofibers packed in a complex matrix of connective tissue. Two main muscle types compose the arm bulk: the longitudinal (L), located at the outer layer, and the transverse (T), surrounding the arm core. Here we investigated the mechanical properties of these muscles using a Dual-Mode Lever System on in-vitro preparations. We show that L and T muscles differ in their biophysics although single myofibers share the same physiological properties. To explore the bases of these different mechanical features, we studied the arm muscle-connective interaction and the possible role of gap junctions in the coordination of muscle ensembles. We performed confocal microscopy of arm sections and employed a waviness index (W.I.) analysis to measure the coiling of elastic fibers. W.I. was found to be higher in L muscles than in T, where elastic fibers are sparsely organized. This architecture contributes to accommodate strain during movements and to coordinate contraction of antagonistic muscles. Moreover, muscle fiber synchrony is achieved despite monosynaptic innervation and might be based on gap junction intercellular connections. Invertebrates gap junctions are formed by innexin, a family of protein analog to vertebrate connexin. As the presence of innexins might be an indicator for gap junction, we sequenced octopus unc-9 innexin and found a good identity and a comparable topology with invertebrate C. elegans unc-9 innexin. The presence of gap junction might suggest that octopus arm muscles evolved together with a fine-tuned coordination control mechanism.

### SESSION 3 SATELLITE CELLS AND MUSCLE REGENERATION IN HEALY MUSCLE AND IN DISEASES

#### 3.1 HDAC4 regulates satellite cell proliferation and differentiation by targeting P21 and Sharp1 genes

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Skeletal muscle exhibits a high capacity to regenerate, mainly due to the ability of satellite cells to replicate and differentiate in response to stimuli. Epigenetic control is effective at multiple steps of this process, regulating the transition from quiescence to activation and differentiation of muscle stem cells<sup>1</sup>. The chromatin-remodelling factor, HDAC4, has been shown to regulate satellite cell proliferation and commitment<sup>2</sup>; however, the underlying molecular mechanisms are still uncovered. To study HDAC4 in satellite cells, we generated a conditional inducible KO mouse line, in which HDAC4 is deleted in Pax7 positive cells, upon tamoxifen administration. Despite having similar amounts of satellite cells, HDAC4 KO mice show compromised satellite cell proliferation and differentiation *in vitro*. To identify the molecular targets regulated by HDAC4 in satellite cells, we performed a ChIP-seq analysis by immuno-precipitating the endogenous HDAC4 in proliferative muscle cells and we performed a RNA-seq analysis by comparing control and HDAC4 KO satellite cells. By intersecting the results of the ChIP-seq with the list of the genes significantly up-regulated in HDAC4 KO satellite cells from the RNA-seq, we identified two candidates, P21 and Sharp1, as direct targets of HDAC4 in proliferating muscle cells. By interfering with their expression, we demonstrated that HDAC4-mediated repression of the cell cycle inhibitor P21, promotes satellite cell amplification; while HDAC4-mediated repression of Sharp1, allows satellite cell differentiation and fusion. These data identify HDAC4-mediated regulation of genes that control two sequential stages of satellite cell activity during myogenesis, such as proliferation and differentiation into new fibers. *References: 1. World J Stem Cells. 2015 Jul 26;7(6):945-55; 2. EMBO Rep. 2014 Nov;15(11):1175-83.*

### 3.2 A first look at Acid Sphingomyelinase in the pathophysiology of Duchenne muscular dystrophy

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Skeletal muscle inflammation is an important feature of different myopathies and supportive evidence for the relevance of inflammation in promoting dystrophin-related diseases come from the beneficial effect of corticosteroids in the treatment of Duchenne muscular

dystrophy (DMD). Although many studies have been focused on the role of bioactive sphingolipids in inflammatory-associated disorders, the involvement of hydrolase acid sphingomyelinase (A-SMase) in DMD-related inflammation is currently unknown. Our results showed that A-SMase is overexpressed in skeletal muscles of mdx mouse model of DMD, both at 4 and 12 weeks of age. The initial inflammatory response during chronic muscle injury is similar to the response to acute damage. To study a possible role of A-SMase in muscle regeneration we injected cardiotoxin in Tibialis Anterioris of wild-type (wt) and A-SMase knock-out (KO) mice thus inducing acute muscle damage. Of interest, the expression of Myogenin, a marker of muscle differentiation, significantly increased in A-SMase-KO mice when compared to wt after cardiotoxin administration. In addition, A-SMase-KO mice muscles showed enhanced levels of the anti-inflammatory cytokines and Arginase, an enzyme which is positively related to muscle regeneration. These data, although preliminary, provide the first evidence that in the absence of A-SMase muscle regeneration is accelerated and suggest a role of A-SMase in the pathophysiology of DMD.

### 3.3 Muscle perivascular stem cells for advanced skeletal muscle tissue regenerative approaches

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Skeletal muscle tissue engineering represents a revolutionary approach for the treatment of musculoskeletal tissue pathologies<sup>1</sup>. However, there are some limitations related to the complex muscle architectural organization and to the difficulty to find a reliable and appropriate source of myogenic progenitors able to sustain swift vascularization. Recent studies have led to the identification of a new muscle progenitor cell population namely pericytes<sup>2</sup>. These are perivascular muscle progenitors able to undergo a robust myogenic differentiation beside a preserved angiogenic ability<sup>3,4</sup>. Human derived pericytes (hPeri) were isolated from human muscle biopsies by enzymatic digestion and selected for low confluence plastic adhesion and alkaline phosphatase (AP) expression<sup>5</sup>, showing colony forming capability and a remarkable spontaneous myogenic activity. hPeri were characterized *in vitro* for mesodermic differentiation and *in vivo* by intramuscular injection, revealing their astonishing myogenic capability. Moreover, for skeletal muscle tissue engineering purpose, it was evaluated the combination of hPeri and PEG-Fibrinogen (PF) based biomimetic scaffold<sup>6</sup> for building a human derived artificial muscle *in vivo* upon subcutaneous

implantation. The results obtained showed that PF based matrix encapsulating hPery promoted the generation of an engineered human derived muscle, moreover presenting an important vascularization upon hPeri angiogenic action. Hence the perivascular compartment with hPeri can be considered a remarkable reliable source for myogenic stem/progenitor cells.

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### 3.4 Mitochondrial bioenergetic profile in correlation with ROS production in young and elderly human satellite cells

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Sarcopenia is defined as the age-related loss of muscle mass, strength and function, associated to regenerative difficulties by satellite cells (SCs), adult muscle stem cells. The oxidative stress and mitochondrial dysfunction are closely linked. The aim of this study was to investigate the metabolic profile of young and aged mitochondria in SCs isolated from human Vastus Lateralis skeletal muscles of young (20-35 y) and elderly (65-80 y) subjects, correlating with ROS production. The mitochondrial superoxide anion (O<sub>2</sub><sup>•-</sup>) production on myoblasts and myotubes was assessed with Mitosox Red Probe. It was performed in the presence of anti-oxidant N-acetylcysteine (NAC) or glucose and sodium pyruvate (burst). Young myoblasts with NAC and burst presented a significant reduction of O<sub>2</sub><sup>•-</sup> levels. In elderly myoblasts, both treatments did not give effects while elderly myotubes increased O<sub>2</sub><sup>•-</sup> levels. The Seahorse Analyzer measures the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). The basal and the maximal OCR in elderly myoblasts were lower respect to the young ones independently from NAC. In presence of NAC elderly myotubes were affected. The spare respiratory capacity and the ATP-linked OCR were impaired only in elderly myoblasts independently from NAC. The glycolytic parameters were higher in young myoblasts compared to elderly ones while an opposite situation observed in myotubes. In conclusion we demonstrated that elderly muscle cells are unable to

handle high glucose concentrations that is associated to impaired mitochondrial function resulting in high ROS levels.

### 3.5 Molecular and cellular mechanisms regulating satellite cell quiescence and growth arrest

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A major challenge in the muscle field is to understand how growth arrest is coordinated in satellite cells (i.e. muscle stem cells) during muscle homeostasis/maintenance and repair. Skeletal muscle shows a remarkable capacity to regenerate after severe injuries, which is attributed to its satellite cell population. Once muscle growth is completed at early postnatal life, this stem cell population enters into a non-cycling, quiescent state. However, in response to specific needs, such as injury, it is rapidly activated to provide differentiated progeny for muscle repair as well as to self-renew the quiescent pool. We have designed a protocol to isolate the satellite cells following direct fixation and defined molecularly the early activation following exit from quiescence. In addition, muscle differentiation is a coordinated process of tissue-specific gene expression and irreversible cell cycle exit. We have analysed the mechanism of growth arrest during terminal differentiation, including the role of Cyclin Dependent Kinase Inhibitors p21 and p57. We will present data regarding p57 and p21 expression and function ex vivo and in vivo, in adult myofiber culture and regeneration models.

### 3.6 Tissue engineering of skeletal muscle for regenerative medicine strategies

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Skeletal muscle is of high clinical interest since many congenital or acquired conditions can affect its function, leading to irreversible loss of tissue – volumetric muscle loss, VML. Tissue engineering aims to mimic neo-organogenesis for producing tissues to be applied in regenerative medicine, and holds great potential for the treatment of incurable skeletal muscle pathologies. Recent translational technologies are based on the use of natural or synthetic scaffolds that ideally can be implanted in patients and aid functional maturation of

skeletal muscle in vivo. In order to investigate the real application of such scaffolds in muscle regenerative medicine, VML mouse models were generated for extensor digitorum longus (EDL) and tibialis anterior (TA) muscles. Decellularised rat EDL were used to rescue the VML of 90% EDL ablation, while a synthetic photo-crosslinkable biomaterial was used for repairing 30% TA ablation. Scaffolds materials were able to be repopulated by recipient cells and supported myogenesis and functional muscle formation. The preservation of native ECM components and 3D organization, strongly improved the ability of the scaffold to support myogenesis.

### 3.7 Macrophages as regulators of skeletal muscle regeneration: factors secreted by murine J774 cells affect proliferation, differentiation and survival of muscle stem cells and fibro-adipogenic progenitors

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Muscle regeneration is a complex, multi-staged process in which macrophages play a fundamental role not only as scavengers of damage tissue but also as modulators of both myogenic and non-myogenic precursors. To study the effects of macrophage-released factors in this context we used the murine macrophage cell line J774 to obtain highly active conditioned medium (mMCM) upon exposure to LPS. We have previously shown that mMCM can enhance the proliferation and differentiation of rat primary myoblasts as well as of normal and dystrophic human myoblasts. We now show that mMCM exert different effects on murine myogenic cells, depending on their physiological state. Specifically, macrophagic factors turned out to have an anti-apoptotic action on freshly isolated satellite cells and a pro-proliferative effect on established cultures of satellite-derived myoblasts. When used to challenge muscle-derived murine fibro-adipogenic precursors, mMCM did not appear to modify their proliferation rate but it showed a potent anti-adipogenic effect, which did not appear to involve the triggering of apoptosis. Interestingly, the effect on FAP differentiation seems to be at least in part related to the exosomes found in mMCM. Lastly, we tested the effects of mMCM on human, muscle-derived pericytes, a population of multi potent precursor cells known to exhibit myogenic

potential. Similarly to what we found in FAPs, mMCM did not affect the proliferation rate of cultured pericytes, but, somewhat surprisingly, it seemed to actually decrease their capability to differentiate towards skeletal muscle cells and form myotubes.

### 3.8 Loss of MMP13 impedes satellite cell migration and delays muscle regeneration

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Skeletal muscle regeneration requires coordinated remodeling of the extracellular matrix (ECM), and matrix metalloproteinases (MMP)s play a critical role. MMP-13, a collagenase, was shown to have high expression during regeneration and regulate C2C12 myoblast migration, yet MMP-13's function in skeletal muscle in vivo has not been studied. Under baseline conditions the Mmp13<sup>-/-</sup> mouse does not exhibit a muscle phenotype; including muscle mechanics, fiber size, capillary density, or ECM area. However, in muscle injured with cardiotoxin, regeneration is delayed at 2 weeks post injury. To determine if these effects were mediated by satellite cell deficiencies when lacking MMP-13, live cell imaging of single fiber cultures were conducted to directly measure migration velocity. Mmp13<sup>-/-</sup> satellite cells had 33% lower migration velocity than controls. In addition, 3D invasion assays through basement membrane extract (BME) revealed that Mmp13<sup>-/-</sup> cells had a 53% reduced ability to migrate through BME substrate. These data extend evidence of MMP-13 being a critical component of myoblast migration in primary cells in 2D and 3D. To further investigate the contribution that MMP-13 mediated migration had on the repair process, we generated a mouse with inducible and satellite cell specific deletion of Mmp13. These mice mirrored many of the phenotypes of the global knockout, including a reduction in satellite cell migration, and delayed resolution of damage by cardiotoxin injection. In sum, MMP-13 mediated satellite cell migration is an important factor for efficient muscle regeneration.

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October 13<sup>th</sup>  
SESSION 4  
GENETIC AND EPIGENETIC ALTERATIONS IN  
MUSCLE DYSTROPHIES AND MYOPATHIES

### 4.1 Histone H3 Lysine 9 methyltransferases G9a and GLP as potential pharmacological targets in skeletal muscle regeneration and Duchenne Muscular Dystrophy

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Histone Lysine Methyltransferases (KMTs) are epigenetic modifiers that dynamically control gene expression during stem cell differentiation. Among the different KMTs, EHMT2/G9a and EHMT1/GLP, responsible of mono- and di-methylation of Lysine 9 of histone H3 (H3K9), are of particular relevance in the context of myogenesis since they have been shown to control the repression of muscle-specific genes in myogenic precursors and to prevent their premature differentiation. Modulation of their activity might therefore be exploited to promote the expression of muscle-specific genes and to enhance muscle differentiation in tissues whose myogenic capacity is compromised due to a pathological condition, as in the case of muscles affected by Duchenne Muscular Dystrophy (DMD). DMD is a severe X-linked neuromuscular degenerative disorder that leads to progressive muscle weakness associated with loss of muscle tissue and replacement with adipose and connective infiltrates, in coincidence with the final stages of disease. Despite recent progresses in genome editing approaches, the cure for DMD is still a big challenge and pharmacological therapies aimed to counteract the fibro-adipogenic degeneration and to promote the compensatory regeneration, typical of the early stages of disease, hold great promise to slow-down DMD progression. Here we provide evidence of the pro-regenerative effect of G9a/GLP specific inhibitors *in vivo*. Our results show that *in vivo* inhibition of G9a/GLP-mediated H3K9me2 improves skeletal muscle regeneration. This is caused by an accelerated myogenic capacity of muscle stem cells (MuSCs) and by an impaired adipogenic differentiation of fibro-adipogenic progenitors (FAPs), which rather unmasks a previously silent myogenic capacity. Our preliminary results provide proof of concept of the use of H3K9 KMTs specific inhibitors as potential pharmacological strategy to promote the regenerative response of diseased, dystrophic, muscles, while concomitantly blocking their fibro-adipogenic degeneration.

#### 4.2 Investigating pathophysiological mechanisms of the heart in DMD

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Several therapies to treat Duchenne Muscular Dystrophy (DMD) are under development; unfortunately many lack efficacy in the heart. Despite the primary genetic defect being identical in skeletal and cardiac muscle, the symptoms and severity differ suggesting the involvement of secondary organ-specific pathways that are yet to be fully understood. We have developed an *in vitro* model of cardiomyocyte hypertrophy using cardiomyocytes isolated from mdx (mouse model of DMD) hearts. Additionally, a transcriptomic approach has been applied to this model, to help elucidate the secondary organ-specific pathways involved. Proteomic analysis of 25 week-old mdx hearts was carried out to identify changes in the early stage of the disease. Using this model, we have been able to utilise various therapeutic approaches to lessen the severity of the phenotype. This model could be a fast, efficient way to screen new and existing approaches for therapeutic efficacy. Restoration of truncated dystrophin protein to these cells via a viral vector rescues the hypertrophic phenotype in addition to the application of pharmaceutical compounds. RNA-Seq data from the hypertrophic cardiomyocytes implicated the involvement of pathways such as angiogenesis, fibrosis and calcium handling. Using data generated from transcriptomics, differentially regulated transcripts were targeted for investigation on a protein level. Data from proteomic analysis of mdx hearts revealed perturbations in the immune response and actin regulation. Identification of genes, proteins and pathways differentially regulated in the mdx heart provides novel insights into the cellular pathophysiology of this organ in DMD and allows the identification of potential therapeutic targets.

#### 4.3 The Polycomb Ezh2 chromatin modifier modulates chronic muscle fibrosis by repressing fibro-adipogenic progenitors' expansion

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In the adult muscle the process of regeneration is necessary to replace and rebuild mature fibers. Following a muscle injury, inflammatory cells and tissue resident fibroblasts are recruited to the site of injury where, among other functions, they contribute to the remodeling of the extra cellular matrix (ECM), a process known as reparative fibrosis. FAPs (fibro-adipogenic progenitors) are thought to be the main source of matrix producing cells in skeletal muscle. In

conditions of chronic or unresolved muscle damage, aging, or muscular dystrophies, FAPs persist and cause excessive scar tissue and/or ectopic fat infiltration, ultimately responsible for the progressive decline of muscle function. Here we identify a novel mechanism by which the Polycomb Ezh2 chromatin modifier modulates the number and proliferation of FAPs within skeletal muscle through the repression of several profibrotic cytokines secreted into the extracellular space. We found that specific deletion of Ezh2 in satellite cells results in the accumulation and hyperproliferation of FAPs in adult and aged muscles. Transcriptome studies showed that component of the TGF $\beta$ , Wnt, TNF- $\alpha$  and PDGF signaling are expressed at higher level in Ezh2 knock out satellite cells, escaping Ezh2 mediated repression and are also found up-regulated in aged satellite cells. Inhibition of these signaling is sufficient to block FAPs proliferation. Importantly treatment of aged FAPs with supernatant isolated from young wild type satellite cells rescue the aged FAPs phenotype, providing further evidence that satellite cells modulate the production of fibrogenic cells in a paracrine fashion. Altogether these results show that epigenetic changes driven by Ezh2 in satellite cells result in functional changes in the muscle niche and point to a role for Ezh2 in regulating persistent fibrosis during aging. Modulation of satellite cells epigenetics and specifically Ezh2 activity may therefore offer an additional therapeutic route for the treatment of chronic fibrosis.

#### 4.4 Gene editing of DMPK mutated gene in myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is the most common adult-onset muscular dystrophy, caused by a (CTG) $_n$  expansion within the 3' untranslated region of the DMPK gene and characterized by progressive myopathy, myotonia and multiorgan involvement. Expression of the mutated gene results in production of toxic transcripts that aggregate as nuclear foci where RNA-binding proteins are sequestered, resulting in mis-

splicing of several transcripts, defective translation and microRNA dysregulation. No effective therapy is yet available for treatment of the disease. In this study we exploited the CRISPR/Cas9 gene editing strategy to remove the pathogenetic repeat expansions for future therapeutic use. To this aim we generated DM1 myogenic cell models from DM1 patient-derived fibroblasts, exhibiting typical disease-associated alterations. We will show that by using the CRISPR/Cas9 and NHEJ gene-editing system CTG expansions can be removed permanently from DMPK gene, resulting in phenotypic reversion of edited cells.

#### 4.5 HDAC4 preserves skeletal muscle structure following long-term denervation mediating the activation of catabolic pathways

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Loss of muscle innervation occurs in several pathological conditions and mainly depends on the imbalance between protein synthesis and degradation (1,2,3). The ubiquitin proteasome system (UPS) and autophagy are the two main proteolytic pathways in skeletal muscle, which are activated upon denervation and contribute to the maintenance of muscle homeostasis (4,5). Histone deacetylase 4 (HDAC4) is an epigenetic factor that mediates skeletal muscle response following denervation. Under this condition, HDAC4 mediates skeletal muscle atrophy (6,7), suggesting the use of HDAC4 inhibitors as pharmaceutical treatment for neurogenic muscle atrophy. Accordingly, mice lacking HDAC4 in skeletal muscle are resistant to neurogenic muscle atrophy. However, the effects of HDAC4 inhibition in a condition of long-term denervation have not been clarified yet. To investigate the role of HDAC4 in skeletal muscle following long-term denervation with a genetic approach, we analyzed mutant mice with a tissue-specific deletion of HDAC4 (HDAC4mKO mice). Strikingly, while HDAC4mKO muscles were resistant to neurogenic muscle atrophy two weeks following denervation, muscles degenerated following 4 weeks of denervation. We demonstrate that, upon denervation, HDAC4 mediates both the UPS and the autophagic flux, which resulted crucial for maintaining muscle homeostasis. Indeed, by triggering either one of these two catabolic pathways in HDAC4mKO mice, muscle degeneration was significantly reduced 4 weeks after denervation. These findings reveal that HDAC4 mediates the activation of the catabolic pathways in skeletal muscle and that inhibition of HDAC4, in a

condition of long-term denervation, leads to skeletal muscle degeneration.

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#### 4.6 An in vitro model of FSHD based on human pluripotent stem cell-derived skeletal myotubes and its application in drug discovery

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Facioscapulohumeral muscular dystrophy (FSHD) is caused by a contraction of the D4Z4 macrosatellite repeat on the subtelomere of chromosome 4. This genetic lesion results in epigenetic derepression and aberrant transcription of the pathogenic Double Homeobox Domain-Containing Protein 4 (DUX4) gene. Understanding the pathogenesis of FSHD and development of a DUX4-silencing therapy will require characterization of the molecular players that direct transcriptional activation of the D4Z4 locus. We developed one of the largest banks of disease-affected human embryonic stem cells (hESCs), including FSHD-affected cell lines. In addition, we developed and published a fast, robust and highly efficient differentiation method for skeletal muscle that does not rely on the overexpression of myogenic transcription factors or cell sorting to enrich myogenic populations (Caron et al. 2016). Together this technology provides a valuable platform to study development and disease states in FSHD with relevant human pathologies that cannot be recapitulated in animal models. It also provides an unlimited, uniform resource to develop cell-based primary and secondary phenotypic assays for drug discovery. Here, we discuss our recent findings on DUX4 expression in stem cell-derived myotubes, its epigenetic regulation and secondary FSHD phenotypes observed in these cells in the context of our overall aim to develop a small molecule drug to treat FSHD patients.

#### 4.7 Novel zebrafish models of sarcoglycanopathy

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Sarcoglycanopathy is the collective name of four rare genetic diseases caused by defects in the genes coding for alpha-, beta-, gamma- and delta-sarcoglycan (SG),

which form a key structural complex that assures sarcolemma stability during muscle contraction. Most of the reported cases of sarcoglycanopathy are due to missense mutations. The resulting folding-defective SG is degraded by the quality control of the cell, leading to the secondary deficiency of the wild type partners. Many missense mutants retain their function as the entire SG-complex can be properly rescued by skipping the degradation of the defective protein. Presently, major effort is devoted to the development of novel therapeutic approaches based on the use of small-molecules either inhibiting the degradation or helping the folding process of SG mutants. Indeed, the application of this strategy allowed the recovery of the SG-complex in both cell models and, notably, primary myotubes from a patient with sarcoglycanopathy.

To confirm in vivo efficacy and tolerability of this successful strategy, we are now generating novel sarcoglycanopathy models in zebrafish, which will carry a mutated SG, recoverable by drug treatment. We are focused on zebrafish because it is an excellent vertebrate model for muscular disorders, drug screening, and it is relatively easy to introduce any desired mutation by genome-editing technologies. Here we report data showing knock-down of delta-SG in zebrafish, that leads to severe muscular abnormalities, well mimicking the human disease, and first results concerning the generation of beta-SG and delta-SG knock-in and knock-out zebrafish lines by the CRISPR/Cas9 technique.

October 14<sup>n</sup>

### SESSION 5 METABOLIC ALTERATIONS AND MUSCLE DISEASES

#### 5.1 Cynidin enriched diet delays muscular dystrophy in alpha-sarcoglycan null mice

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Muscular dystrophies (MD) are genetic diseases, all characterized by a progressive muscle wasting in time, leading patients to wheelchair and premature death caused by respiratory and cardiac failure. Nowadays there is still no definitive cure for these pathologies. Only chronic treatment of corticosteroids is widely used to slow down the symptoms, but with several side effects for the patients. It is also known the myofibers affected by MD are more susceptible to oxidative stress than healthy fibers due to a lacking of antioxidant signal. In this study we investigate on the effects of a diet enriched with a natural antioxidant (Cyanidin, red diet RD) on the dystrophic mouse model *Sgca* null. We

observed a delay on the typical markers of the MD onset, as more homogeneous CSA distribution, reduction of fibrosis and also a rescue in the muscle performance. We also addressed the molecular pathways triggered by RD, identifying an exclusion of the transcription factor NF- $\kappa$ B from the myonuclei as an anti-inflammatory pathway. On the other side we found that RD promotes Nrf-2 translocation into the nuclei to trigger an antioxidant response through AMPK activity.

### 5.2 High intensity exercise induced muscle remodelling requires histone modifications and increased MRTF-SRF signaling

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Gene transcription is strongly influenced by various posttranslational modifications of the N-terminal tails of histones. The aim of our study is to understand which role histone modifications play during exercise-induced chromatin remodelling in skeletal muscle. In our analyses, we found a rapid and significant increase in phosphorylation of Histone 3 on Serine 10 after eccentric contractions. Moreover, we have found that this histone phosphorylation is followed by an increase in the acetylation of the nearby lysine 14, suggestive of an activation of gene transcription.

We identified p38-MSK1, whose activity is strongly up regulated during exercise in muscles, as the kinase involved in this phosphorylation. We are now investigating the upstream signals responsible for p38-MSK1 activation and the histone modifications observed. In addition to our results in mice, we confirmed our results in human biopsies taken immediately after high intensity exercise where we have observed similar changes in signaling and histone phosphorylation. In our model of exercise, the activation of the early genes FOS, JUN and EGR1 have been confirmed by CHIP analyses on the phosphorylated histone. We are now exploring the expression of immediate early genes in MSK1/2 ko mice to understand the importance of histone phosphorylation in the early gene response to exercise.

### 5.3 The differentiation of fibro adipogenic progenitors of a mouse model of Duchenne Muscular Dystrophy is insensitive to negative regulation by Notch

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Fibro adipogenic progenitors (FAPs) play a leading role in muscle regeneration by positively regulating satellite cells differentiation. However, in pathological conditions, they are responsible for fibrosis and fat infiltration. Despite the established importance of FAPs in both regeneration and degeneration, the signals that regulate these opposing roles are not fully characterized. Our results support a model whereby Notch plays an important role in the regulation of FAP differentiation. In addition, when co-cultured ex vivo, myotubes inhibits the adipogenic differentiation of FAPs in a Notch dependent way. Interestingly, this control mechanism is impaired in FAPs isolated from young dystrophin-deficient (mdx) mice or in FAPs from ageing wild type mice. To further investigate these phenotypic differences, we performed deep proteomics profiling and multiparametric analyses of mdx and wild type FAPs, by exploiting single cell mass cytometry and mass spectrometry based proteomics. Both analyses reveal clear differences in the phenotypes of the mdx and wild type cell populations and in their proteome profiles. Significantly we observed a striking perturbation of the concentration level of metabolic enzymes where a significant decrease of enzymes of the TCA cycle and fatty acid oxidation pathway was balanced by an increase of the enzymes of the glycolytic pathway. In addition an increase in proteins involved in exosome formation or in response to activation of toll-like receptors was also observed.

### 5.4 Lamin A/C is crucial for skeletal muscle plasticity

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Mutations in the LMNA gene encoding the nuclear lamins A/C cause a variety of diseases, including lamin-related congenital muscle dystrophy (L-CMD). The underlying mechanisms by which LMNA mutations cause tissue specific disease remain elusive. Given the apparent contribution of lamins A/C to cellular mechanotransduction, we hypothesized that defective mechanical properties of LMNA mutated muscle

contribute to the dystrophic phenotype in L-CMD. To assess muscle plasticity, plantaris muscle (PLN) of WT and LMNA+/ $\Delta$ K32 mice were mechanically overloaded by synergy ablation of the gastrocnemius and soleus muscles. PLN muscles were analyzed 1 or 4 weeks after mechanical overload. In addition, human derived myoblasts carrying the same mutation in the lamin A/C gene were differentiated into myotubes and subjected to cyclic stretch. In response to 1 and 4 weeks of mechanical overload, WT PLN mass/body mass increased 1.9 and 2.3 fold respectively compared to control WT, but LMNA+/ $\Delta$ K32 mice failed to produce a comparable hypertrophic response (1.1 and 1.6 fold increase respectively compared to WT controls). Paradoxically, anabolic signals (Akt/P70S6K) were similarly activated between mouse strains, however the LMNA+/ $\Delta$ K32 mice displayed a significant up regulation of both total and de-phosphorylated FoxO3a and its downstream gene targets (Murf1, MAFbx, Musc1, Fbxo31, SMART). The average number of Pax7+ cells per cross-section and Pax7 protein expression were significantly lower in LMNA+/ $\Delta$ K32 PLN compared to WT. In addition, we observed a high rate of fragmentation of the neuromuscular junction (NMJ) following overload whereas WT PLN were characterized by NMJ hypertrophy. In vitro data showed that myotube morphology was markedly different between cell types with increased myotube size, nuclei per myotube and cytoskeletal disorganization observed in LMNA+/ $\Delta$ K32 vs WT myotubes. Acute mechanoresponsiveness of LMNA+/ $\Delta$ K32 myotubes was aberrant in vitro as determined by abnormal localization and activity following stretch of the mechanosensitive proteins YAP and MKL1 accompanied by a lack of cytoskeleton reorganization in LMNA+/ $\Delta$ K32 myotubes. In conclusion, our results show that the muscle ability to sense and respond to mechanical overload is severely disturbed in L-CMD. These data highlight the importance of the nuclear envelope in mechanosensing, shedding light on the intrinsic force sensing mechanisms in skeletal muscle.

### 5.5 Monitoring oxygen-sensors in cardiac hypertrophy using a non-pressure overload animal model

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Met Activating Genetically Improved Chimeric Factor 1 (Magic-F1) is a human recombinant protein, derived from dimerization of the receptor-binding domain of hepatocyte growth factor (HGF). Previous experiments demonstrate that in hemizygous transgenic mice, the skeletal muscle specific expression of Magic-F1 can induce a constitutive muscular hypertrophy, improving running performance and accelerating muscle regeneration after injury [1]. Furthermore, the microarray analysis of Magic-F1+/+ satellite cells evidenced transcriptomic changes in genes involved in the control of muscle growth, development and vascularisation [2]. In this study we demonstrate that Magic-F1 mice show an alteration of the heart morphology. Data obtained by morphometric analysis and three-dimensional reconstruction of the heart revealed that circulating Magic-F1 proteins are able to induce a dilatation of the left ventricle chamber of transgenic mice. Interestingly, we found in Magic-F1 hearts an alteration of Phd2 and HIF1 protein levels. These two oxygen sensors are found dysregulated in cardiac ischaemic conditions, where generalised hypoxia causes functional impairments in cardiomyocytes and structural tissue damage [3-4]. These preliminary results support the involvement of oxygen sensors in Magic-F1-induced cardiac hypertrophy. In addition, Magic-F1+/+ mice can be used as non-pressure overload model to further investigate the role of oxygen-sensors in ischaemic heart disease. References: 1. Cassano M et al. (2008). Magic-factor 1, a partial agonist of Met, induces muscle hypertrophy by protecting myogenic progenitors from apoptosis. PLoS One. 2008 3(9): e3223. 2. Ronzoni F et al. (2017). Met-Activating Genetically Improved Chimeric Factor-1 Promotes Angiogenesis and Hypertrophy in Adult Myogenesis. Curr Pharm Biotechnol. 2017 Feb 1. 3. Di Conza G et al. (2017). The mTOR and PP2A Pathways Regulate PHD2 Phosphorylation to Fine-Tune HIF1 $\alpha$  Levels and Colorectal Cancer Cell Survival under Hypoxia. Cell Rep. 2017 Feb 14. 4. Piccoli M et al (2016). A chemical approach to myocardial protection and regeneration. Eur Heart J Suppl. 2016 Apr 28.

### 5.6 A MICU1 splice variant confers high sensitivity to the Ca<sup>2+</sup> uptake machinery of skeletal muscle

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Skeletal muscle is a dynamic organ, characterized by an incredible ability to rapidly increase its rate of energy consumption to sustain activity. Muscle mitochondria provide most of the ATP required for contraction via oxidative phosphorylation. Here, we found that skeletal muscle mitochondria express a unique MCU complex containing an alternative splice isoform of MICU1, MICU1.1, characterized by the addition of a micro-exon that is sufficient to greatly modify the properties of the MCU. Indeed, MICU1.1 binds Ca<sup>2+</sup> one order magnitude more efficiently than MICU1 and, when heterodimerized with MICU2, activates MCU current at lower Ca<sup>2+</sup> concentrations than MICU1-MICU2 heterodimers. In skeletal muscle in vivo, MICU1.1 is required for sustained mitochondrial Ca<sup>2+</sup> uptake and ATP production. These results highlight a novel mechanism of the molecular plasticity of the MCU Ca<sup>2+</sup> uptake machinery that allows skeletal muscle mitochondria to be highly responsive to sarcoplasmic [Ca<sup>2+</sup>] responses.

**October 14<sup>th</sup>**  
**SESSION 6**  
**THERAPEUTIC APPROACHES FOR MUSCLE DISEASES**

### 6.1 The voice of patients and their families – Parent Project

### 6.2 Improving the myogenic regenerative capacity of induced pluripotent stem cell-derived mesodermal progenitors

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Pluripotent stem cells (PSCs) have a high proliferative capacity and can give rise to all three embryonic germ layers. Due to these characteristics, PSCs have been extensively researched in the field of muscular dystrophies as a large number of cells is required to restore the entire muscle pool. Recently, our group has successfully isolated induced PSC-derived mesodermal progenitors (MiPs) that can regenerate both cardiac and skeletal muscle<sup>1</sup>. Nevertheless, improvements need to be made to work towards a clinical relevant protocol that has an efficacy high enough to allow functional improvements. First of all, MiPs are obtained through

the formation of embryoid bodies using serum-containing media thereby limiting their clinical translatability. Secondly, although MiPs showed to contribute to both cardiac and skeletal muscle, functional improvement was only moderate. For this study, a serum-free monolayer approach, adapted from Shelton et al. (2014), was used to derive mesodermal progenitors from induced PSCs in a clinical relevant manner<sup>2</sup>. These progenitors were tested in vitro for their potential to contribute to the myogenic lineages. Next, in order to further enhance their myogenic capacity and later engraftment, stimulation of the Dll1/Notch1 signaling pathway is being explored as this has been shown to promote the myogenic commitment of adult stem cells<sup>3</sup>. With the purpose of cell therapy in mind, two small molecules, namely valproic acid and resveratrol, are being investigated for their potential to improve the myogenic capacity of the obtained mesodermal progenitors and whether this effect can be attributed to the stimulation of the Dll1/Notch1 pathway. References: Quattrocelli, M. et al. Mesodermal iPSC-derived progenitor cells functionally regenerate cardiac and skeletal muscle. *J. Clin. Invest.* 125, 4463–82 (2015). 2. Shelton, M., Kocharyan, A., Liu, J. & Skerjanc, I. S. Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells. *Methods* 101, 73–84 (2016). 3. Quattrocelli, M., Costamagna, D., Giacomazzi, G., Camps, J. & Sampaolesi, M. Notch signaling regulates myogenic regenerative capacity of murine and human mesoangioblasts. *Cell Death Dis.* 5, e1448 (2014).

### 6.3 Sarcoglycanopathy: from the molecular mechanism to new therapeutic perspectives

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Sarcoglycanopathy is caused by mutations in sarcoglycans (SG), four glycoproteins that form an essential complex for the integrity of muscle cells. Most sarcoglycan mutations are missense mutations generating a folding defective protein, which is prematurely degraded by the cell's quality control. We recently unveiled the pathway responsible for the disposal of defective alpha-SG and demonstrated that many missense mutants retain their function. Indeed, the complex can be properly rescued by targeting the degradative pathway. Thanks to these knowledges, we design a novel therapeutic approach for sarcoglycanopathy based on the use of small molecules able to improve the folding process of defective sarcoglycans. Once structurally stabilized, they can skip disposal and traffic at the proper site of action. To this intent, we tested compounds of the library of the CFTR

correctors and several of them were effective in rescuing SG mutants in cell models and, notably, in myotubes from a patient with alpha-sarcoglycanopathy. Although we need to clarify the mechanism by which CFTR correctors exert their activity in sarcoglycanopathy, these data represent the proof of principle of a novel pharmacological strategy for this, still incurable, disease. Now we are focused on the development of unconventional animal models for in vivo studies, essential to confirm efficacy as well as safety and tolerability of these compounds. At present, we are characterizing at the histological and molecular level, mouse models obtained by the AAV-mediated gene delivery of mutated versions of the human sequence in the null alpha-SG background.

#### **6.4 Human dental pulp pluripotent-like stem cells promote tissue regeneration through paracrine signaling and direct contribution**

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Dental pulp pluripotent-like stem cells (DPPSC) isolated from human third molar pulp express embryonic stem cell markers<sup>1</sup> and show pluripotent-like behavior,<sup>2</sup> making DPPSC an appealing tool for tissue repair or maintenance. In this study, DPPSC obtained from young patients were characterized and their secretome was analyzed. We then evaluated DPPSC differentiation potential towards endothelial, smooth and skeletal muscle lineages. The in vivo contribution of DPPSC was tested in a wound-healing mouse model and in two immuno-deficient mice resembling Duchenne muscular dystrophy and limb-girdle muscular dystrophy type 2E (Scid/mdx and Sgcb-null Rag2-null  $\gamma$ c-null [3], respectively). Our results showed that DPPSC secreted several growth factors involved in angiogenesis and extracellular matrix deposition and that DPPSC treatment improved vascularization in all mouse models. In dystrophic mice, DPPSC integrated in muscular fibres and vessels, and induced larger cross-

sectional area of type II fast-glycolytic fibres. In addition, DPPSC treatments resulted in reduced fibrosis and collagen content and changes in macrophage polarization. This is likely due to the observed cytokine profile modification, with higher levels of interleukin-10 in DPPSC-injected muscles compared to controls. Overall, DPPSC represent a source of stem cells with the potential to enhance wound healing and slow down dystrophic muscle degeneration. References: 1. Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferrer Padro E, Giner L, Casals N: Isolation of pluripotent stem cells from human third molar dental pulp. *Histology and histopathology* 2011, 26(8):1057-1070. 2. Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F et al: Dental pulp of the third molar: a new source of pluripotent-like stem cells. *Journal of cell science* 2012, 125(Pt 14):3343-3356. 3. Quattrocchi M, Swinnen M, Giacomazzi G, Camps J, Barthelemy I, Ceccarelli G, Caluwe E, Grosemans H, Thorrez L, Pelizzo G et al: Mesodermal iPSC-derived progenitor cells functionally regenerate cardiac and skeletal muscle. *The Journal of clinical investigation* 2015, 125(12):4463-4482.

#### **6.5 Strengthening the neuromuscular junction as a new concept for the treatment of congenital myasthenic syndromes and motor neuropathies with synaptic dysfunction**

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Disruption of the development and maintenance of the neuromuscular junction (NMJ) through mutations in the genes encoding its components lead to congenital myasthenic syndromes (CMS). These disorders result in impaired neurotransmission and muscle weakness; current treatments for these conditions are limited. Development and maintenance of the NMJ depends on the neuronal factor agrin and its downstream pathway. Neurotune AG has developed a modified form of agrin (NT-1654) which has been shown to stimulate the NMJ development pathway. The aim of this project is to test NT-1654 in mouse models of human CMS, and provide evidence for its use as a therapeutic compound in humans. We have conducted dose finding studies to identify safe, non-toxic concentrations of the drug in three animal models of CMS: DOK-7(c.1124\_1127dupTGCC), COLQ(-/-), and Agrin (nmf380), and one of hereditary neuropathy:

GARS(C201R). Daily subcutaneous injections of either 1mg/kg, 5mg/kg, 10mg/kg, or PBS, were administered and animals monitored for signs of adverse reactions and toxic effects. So far all animals have all tolerated the drug well, with no adverse changes being noticed. Full studies administering NT1654 have now been initiated in two of the CMS models with further models expected to begin soon. Preliminary data regarding body weight, grip strength, swallowing ability, and survival will be presented. It is hoped that these investigations will provide the rationale for beginning human trials of NT1654 in patients.

### 6.6 Autologous intramuscular transplantation of engineered satellite cells induces exosome-mediated systemic expression of Fukutin-Related Protein and rescues disease phenotype in a murine model of Limb-Girdle Muscular Dystrophy Type 2I

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$\alpha$ -Dystroglycanopathies are a group of muscular dystrophies characterized by  $\alpha$ -DG hypoglycosylation and reduced extracellular ligand-binding affinity. Among other genes involved in the  $\alpha$ -DG glycosylation process, fukutin related protein (FKRP) gene mutations generate a wide range of pathologies from mild limb girdle muscular dystrophy 2I (LGMD2I), severe congenital muscular dystrophy 1C (MDC1C), to Walker-Warburg Syndrome and Muscle-Eye-Brain disease. FKRP gene encodes for a glycosyltransferase that in vivo transfers a ribitol phosphate group from a CDP-ribitol present in muscles to  $\alpha$ -DG, while in vitro it can be secreted as monomer of 60kDa. Consistently, new evidences reported glycosyltransferases in the blood, freely circulating or wrapped within vesicles. Although the physiological function of blood stream glycosyltransferases remains unclear, they are likely released from blood borne or distant cells. Thus, we hypothesized that freely or wrapped FKRP might circulate as an extracellular glycosyltransferase, able to exert a "glycan remodelling" process, even at distal compartments. Interestingly, we firstly demonstrated a

successful transduction of MDC1C blood-derived CD133+ cells and FKRP L276IKI mouse derived satellite cells by a lentiviral vector expressing the wild-type of human FKRP gene. Moreover, we showed that LV-FKRP cells were driven to release exosomes carrying FKRP. Similarly, we observed the presence of FKRP positive exosomes in the plasma of FKRP L276IKI mice intramuscularly injected with engineered satellite cells. The distribution of FKRP protein boosted by exosomes determined its restoration within muscle tissues, an overall recovery of  $\alpha$ -DG glycosylation and improved muscle strength, suggesting a systemic supply of FKRP protein acting as glycosyltransferase.

October 14<sup>th</sup>

## SESSION 7

## MUSCLE WASTING, SARCOPENIA AND CACHEXIA

### 7.1 Inhibition of glutathione peroxidase 4 primes mouse C2C12 myoblasts and rhabdomyosarcoma cell lines to ferroptosis

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Ferroptosis is a recently discovered form of cell death causally linked to the ability of iron to induce oxidative damage by peroxidation of polyunsaturated fatty acids (PUFAs). Misregulated ferroptosis has been implicated in a number of pathological processes and there is a growing interest in the pre-clinical use of ferroptosis inducers against tumors. Cells to prevent ferroptosis mostly engage in the activity of glutathione peroxidase 4 (GPx4), a selenoenzyme that uses glutathione for neutralizing lipid hydroperoxides. Two major ferroptosis inducers mediating GPx4 inhibition have been identified, namely Erastin (eradicator of RAS and ST-expressing cells) and RSL3 (RAS selective Lethal 3). In this work we have investigated their effect on mouse skeletal C2C12 myoblasts and cell lines of rhabdomyosarcoma (RMS), the most frequent soft-tissue tumor affecting children and adolescents. As evaluated by using specific fluorescent probes, treatment with Erastin or RSL3 agents resulted in a marked production of both cytoplasmic/mitochondrial ROS and lipid ROS, which correlated in a dose-dependent manner with a decreased cell viability, as evaluated by means of Neutral Red assays after 48 hours. In Erastin-treated cell lines ferroptosis was enhanced in the presence of iron supplementation (through ferric ammonium citrate), while it was prevented by pre-treatment with agents sequestering iron (bathophenanthrolinedisulfonic acid), antioxidant

scavengers (glutathione and N-acetylcysteine) and lipid ROS scavengers (ferrostatin-1). We observed Erastin to be more effective to promote ferroptosis in the cell lines showing a higher proliferation rate. Indeed, inhibition of ERK signaling, as observed during differentiation or upon pharmacological treatment with PD090859 agent, prevented ferroptosis in Erastin-treated human RMS embryonal RD and C2C12 cell lines. Furthermore, we found Erastin and RSL3 to be more effective in inducing ferroptosis in RD subclones characterized by higher ERK1/2 phosphorylation and proliferation rate. Taken together, our data suggest that iron metabolism could play a key role in the cell fate of muscle cells; in addition, the use of ferroptotic inducers could offer a novel alternative to improve the efficacy of conventional antineoplastic cocktails utilized against RMS.

### 7.2 Micro-computed tomography for non-invasive evaluation of muscle atrophy in mouse models of neuromuscular disease and cancer cachexia

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Muscle wasting occurs during various chronic diseases and precedes death in humans as in mice. Assessing the degree of muscle atrophy in diseased mouse models is often overlooked since it requires the sacrifice of the animals for muscle examination or expensive instrumentation and highly qualified personnel, such as Magnetic Resonance Imaging (MRI). We developed a non-invasive procedure based on micro-computed tomography (micro-CT) without contrast agents to monitor hind limb muscle wasting in mouse models of neuromuscular disease and cancer cachexia: the transgenic SOD1G93A mouse and the colon adenocarcinoma C26-bearing mouse, respectively. We established the scanning procedure and the parameters to consider in the reconstructed images to calculate the Index of Muscle Mass (IMM). We performed longitudinally micro-CT scan of hind limbs in SOD1G93A mice at presymptomatic and symptomatic stages of the disease and calculated the IMM. We found that IMM in SOD1G93A mice was lower than age-matched controls even before symptom onset. We also detected a further decrease in IMM as disease progresses, most markedly just before disease onset. We

performed the same analyses in the C26-based mouse model, losing progressively body and muscle mass because of cachexia. Interestingly, we found a strong correlation between IMM and Tibialis Anterior and Gastrocnemius muscle weights in both disease models. We developed a fast, easy-to-conduct and cost-effective imaging procedure to monitor hind limb muscle mass useful in preclinical therapeutic trials but also in proof-of-principle studies to identify the onset of muscle wasting. This procedure could be widely applied to other disease models characterized by muscle wasting, to assist drug development and search for early biomarkers of muscle atrophy.

#*Equally contributing authors.*

### 7.3 Musclin: an exercise-induced myokine useful to contrast muscle wasting during cancer

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Physical activity extends life span of patients affected by certain types of cancer, also by contrasting the associated muscle wasting (i.e. cachexia). The most effective type of physical activity against muscle wasting during cancer seems to be aerobic exercise. So, we asked whether it promotes secretion of proteins by muscles (i.e. myokines) that may contrast cancer cachexia. To mimic aerobic exercise, we infected C2C12 myotubes with PGC1 $\alpha$ -expressing adenoviruses, because PGC1 $\alpha$  is the main transcriptional coactivator involved in muscle adaptation during aerobic exercise. Our microarray analysis showed musclin as a PGC1 $\alpha$ -induced myokine. We further immunoprecipitated it only from supernatants of PGC1 $\alpha$  expressing-myotubes. By Q-PCR, we found musclin expression unchanged in myotubes hypertrophy because of activated AKT (to mimic anaerobic exercise). Among other PGC1 $\alpha$ -induced myokines, we found only musclin strongly downregulated in cachectic muscles and plasma of C26-bearing mice even at times when their body weights were not lost yet. Thus, we electroporated Tibialis Anterior (TA) of C26-bearing mice with musclin-encoding plasmids and found musclin to preserve fiber area. Dexamethazone-treated myotubes or FoxO3-expressing myotubes undergo atrophy as measured by increased rates of proteolysis and MuRF1 induction. Unlike GFP, musclin was able to contrast the dexamethazone induced-MuRF1 expression in Luciferase assays. Notably, musclin-containing supernatants of PGC1 $\alpha$  expressing-myotubes restrained the FoxO3-induced rates of long-lived protein degradation. Musclin is a myokine induced specifically by PGC1 $\alpha$ , typically increased upon aerobic exercise

and musclin overexpression is beneficial against muscle wasting during C26 growth or in atrophying myotubes. Overall, musclin would be a good drug option for cancer patients that cannot exercise and are at risk of developing cachexia.

#### 7.4 Elderly human mesoangioblasts are impaired in proliferation and in differentiation potential

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Muscle wasting is responsible for severe debilitating weakness and it is well known as sarcopenia when it occurs in aged people causing loss of muscle mass, strength and function [1]. Recent literature revealed that human satellite cells are impaired in elderly subjects [2], however, how interstitial muscle cells affect tissue regeneration and fatty deposition upon ageing has not yet been elucidated. Firstly, we isolated the non-satellite cell fraction from human muscle biopsies of young and elderly subjects, referred here as CD56- interstitial cells. The elderly CD56- interstitial cells showed a larger number of CD15+ and PDGFR $\alpha$ + cells in comparison with young interstitial cells. Moreover, CD56-/ALP+ cells were the most abundant plastic cell population able to differentiate into smooth muscle cells and adipocytes, and the unique interstitial population with myogenic differentiation potential. They expressed pericyte markers including NG2,  $\alpha$ SMA and PDGFR- $\beta$  and were referred as mesoangioblasts (MABs), vessel-associated stem cells. Interestingly, elderly MABs displayed a dramatic impairment in the myogenic differentiation ability in vitro and after transplantation in immunodeficient dystrophic mice (Sgcb-null Rag2-null  $\gamma$ c-null mice). In addition, elderly MABs proliferated less, but yet retained other multilineage capabilities. Taken together, our results suggest that the CD56- cell fraction is modulated in skeletal muscle during ageing, it is possibly involved in the fibroadipogenic deposition and could represent a feasible target for future treatments aimed to reduce muscle loss and wasting. References: 1) Delbono O (2011). Expression and regulation of excitation-contraction coupling proteins in aging skeletal muscle. *Current aging science*. 4, 248-259. 2) Pietrangelo T, Di Filippo ES, Doria C, Rotini A,

Fanò-Ilicic G, Fulle S (2015). Low Intensity Exercise Training Improves Skeletal Muscle Regeneration Potential. *Frontiers in Physiology* 6:399.

#### 7.5 Role of MICAL2 in adult myogenic differentiation

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The dystrophin-associated glycoprotein complex (DGC) serves as a mechanical link between the cytoskeleton and the extracellular matrix of muscle cells. Disassembly of this protein complex renders the sarcolemma vulnerable to contraction-induced injury, leading to progressive fiber damage, membrane leakage and cell death. DGC interacts with Filamentous-actin (F-Actin) fine regulated by Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 2 (MICAL2). Indeed, MICAL2 modifies actin subunits and promotes actin filament turnover by severing disaggregation and preventing repolymerization. MICAL2 has been shown to transduce semaphorin/plexin external signaling into cytoskeletal modifications [1]. Interestingly, in a genome profiling study, MICAL2 has been found among a set of ten functionally linked genes involved in muscle degenerations of mdx mice [2]. In this study, we focus on the role of MICAL2 in skeletal, cardiac and smooth muscle differentiation. In particular, we found that MICAL2 increases during myogenic differentiation of C2C12 cells and primary satellite cells. Intriguingly, perturbation studies of MICAL2 levels impair myogenic differentiation, both in C2C12 and satellite cells. Murine mesoangioblasts, vessel associated stem cells, also express high levels of MICAL2 when differentiated into smooth muscle cells. Finally, murine hearts express MICAL2 protein both in embryonal and adult conditions, as well as human induced pluripotent stem cells during differentiation into cardiomyocytes. Taken together, these data demonstrate that modulations of MICAL2 have an impact on muscle differentiations. Further experiments are necessary to understand whether the absence of MICAL2 affects smooth and cardiac muscle differentiation. Moreover, gain-of-function experiments might shed light on the role of MICAL2 in myogenic commitments. References: 1. Lundquist et al. (2014) Redox modification of nuclear actin by MICAL-2 regulates SRF signaling. *Cell*. 156(3):563-76. 2. Marotta et al. (2009) Muscle genome-

wide expression profiling during disease evolution in mdx mice. *Physiol Genomics*. 37(2):119-32.

### 7.6 25(OH) Vitamin D3 protects from atrophy in vitro

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Cachexia is a common complication of cancer characterized by several metabolic alterations and a massive skeletal mass loss occurring in up to 60% of cancer patients. In addition to increasing morbidity and mortality, aggravating the side effects of chemotherapy, and reducing quality of life, cachexia is considered the direct cause of death of a large proportion of cancer patients. However, no efficacious treatment exists today. 25(OH) vitamin D3 (25OHD) blood levels have been correlated with the incidence and evolution of some cancers, suggesting that vitamin D (VD) can play a role in improving patients' prognosis. VD has been also shown to be important in the maintenance of muscle homeostasis and functionality. This led to the hypothesis of using VD as anti-cachectic treatment, but the direct effect of VD supplementation on muscle and its mechanism are still not clear. Here we show that 25OHD protects C2C12 myotubes from cytokine-induced atrophy by activating Akt-FOXO3 signaling, and that the intracellular conversion of 25OHD to 1,25(OH)<sub>2</sub> vitamin D3 is crucial for its anti-atrophic function. Moreover, 25OHD treatment promotes hypertrophy. Altogether, our data indicate that 25OHD has a protective effect on skeletal muscle cells in vitro. Thus, 25OHD could be a possible candidate for further studies in cancer-cachexia treatment.

**POSTER SESSION:  
ALWAYS ON DISPLAY**

### P.01 Evaluation of anti-atrophic activity of anamorelin in models of muscle wasting

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Cachexia is a metabolic syndrome characterized by an involuntary loss of skeletal muscle and adipose tissue that leads to progressive functional impairment. This syndrome affects about 50-80% of cancer patients and there are no standard therapy regimens for its management. Acylated and unacylated ghrelin (AG and UnAG) are circulating peptide hormones generated mainly in the stomach due to fasting or caloric restriction. AG, through binding to GHSR-1a, induces strong GH release, stimulates food intake, adiposity, and positive energy balance. Acylation of ghrelin is essential for its binding to GHSR-1a, since UnAG does not activate this receptor. In both patients and animal models, AG ameliorates cachexia induced by several pathological conditions in a GHSR-dependent manner. However, we have shown that both AG and UnAG directly protect skeletal muscle from experimentally-induced atrophy, independently of GHSR-1a, thus providing the evidence for the existence of an alternative ghrelin receptor. Anamorelin (ANAM) is a selective agonist of GHSR-1a with appetite-enhancing and anabolic effects. Different clinical studies have highlighted its ability to improve the cachectic state in cancer patients by increasing total body mass. Nevertheless, the effects of ANAM on muscle tissues have not yet been completely elucidated. The data herein presented show that ANAM not only binds to GHSR-1a, but also recognizes the alternative receptor responsible for the anti-atrophic activity shown by ghrelin. Indeed, ANAM protects C2C12 myotubes from experimentally-induced atrophy and activates protein synthesis. These effects are GHSR-1a independent, since they are also visible in culture of primary myotubes obtained from GHSR-1a knock-out mice.

### P.02 Glycosylation stabilizes IGF-1Ea pro hormone and regulates its secretion

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Insulin-like growth factor-1 (IGF-1) is a growth factor with multiple roles in various aspects of normal and pathological growth and differentiation. The translation of the IGF-1 gene gives rise to an immature IGF-1 peptide, which has a signal peptide at the 5' end of the gene, a core region and an Ea-peptide at the 3' end. The signal peptide is removed after facilitating the passage of the polypeptide into the endoplasmic reticulum and give rise to the IGF-1 prohormone (proIGF-1Ea), retaining C-terminal Ea peptide. Recent studies demonstrated that intracellular IGF-1 is mainly

expressed as prohormone, not mature IGF-1. Moreover, we recently demonstrated that the Ea peptide is an intrinsically disordered region (IDR) enriched in regulatory elements including a highly conserved N-glycosylation site. In this study we investigate the role of Ea peptide glycosylation on proIGF-1Ea stability and secretion. After transient transfection of Hek293 cells with IGF-1Ea transgene two IGF-1 prohormones were produced intracellularly: glycosylated (~17kDa) and non-glycosylated forms (~11-12kDa). Subsequently, we wondered whether glucose withdrawal or direct inhibition of N-glycosylation by tunicamycin (Tun) might interfere with IGF-1Ea production. To achieve this aim, we overexpressed proIGF-1Ea in Hek293 cells cultured in glucose depleted medium or treated with Tun. Notably, the band corresponding to glycosylated proIGF-1Ea completely disappeared in the absence of glucose or after treatment with Tun. Moreover, the analysis of culture media of IGF-1Ea-transfected Hek293 cells showed that the inhibition of glycosylation by glucose deprivation or Tun completely abrogated the glycosylated proIGF-1Ea secretion and markedly reduced the mature IGF-1 secretion. After that, using the protein synthesis inhibitor cycloheximide, we demonstrated that the turnover rate for non-glycosylated IGF-1Ea was faster than glycosylated IGF-1Ea. To test the involvement of 26S proteasome machinery, we subsequently treated IGF-1Ea-transfected Hek293 cells with proteasome inhibitor MG132 and we found an increase of non-glycosylated IGF-1Ea, while IGF-1Ea glycosylated was marginally affected by proteasome inhibitor. In conclusion, these results show that glycosylation of Ea peptide enhances the export efficiency of proIGF-1Ea and it is necessary for IGF-1 secretion. We hypothesize that proIGF-1Ea glycosylation ensures proper prohormone folding and secretion preventing its entry into the ER-associated degradation (ERAD) pathway.

### P.03 2D and 3D Disease Modeling of Becker and Duchenne's Muscular Dystrophies

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Duchenne and Becker muscular dystrophies (D/BMD) are caused by a variety of mutations in the dystrophin gene and collectively comprise the most prevalent congenital skeletal muscle disorders. Although a recent therapeutic treatment has been approved by the FDA, long term therapeutic benefits are pending and subpopulations of D/BMD patients do not benefit from it. Drug discovery in D/BMD, as well as other orphan disorders, is typically hampered by the lack of adequate

disease models, clinically irrelevant assays or a combination of both. Herein, we present a human pluripotent stem cell (hPSC) derived two- and three-dimensional (2D and 3D) D/BMD disease modeling platform, amenable to use with hPSCs carrying any type of dystrophin mutations. Skeletal muscle differentiation is achieved using Genea Biocells' simple three media process as previously described by Caron et al. (*Stem Cell Trans Med*, 2016). For 2D modeling, monolayer hPSCs are plated onto collagen I-coated dishes and differentiated through three myogenic developmental stages (e. g. satellite-like cells, myoblasts and myotubes), generating cultures suitable for morphological and metabolic analyses and cell-based drug screening assays. For 3D modeling, similar to previously published work using primary human myoblasts (Madden et al., *eLife*, 2015), hPSC-derived myoblasts generated in the 2D system were encapsulated in hydrogel based myobundles to promote differentiation into myotubes using a 3D culture environment. The 3D myobundles are suitable for histology, as well as contractile force measurements, both critical clinical outcome measures. Collectively, these platforms provide a patient-specific disease-in-a-dish model with clinically-relevant assay endpoints for drug discovery programs. Moreover, this approach can be extended to model a variety of other skeletal muscle disorders.

### P.04 Role of IGF-1 in sarcopenia

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Sarcopenia is the progressive aging-related loss of skeletal muscle mass and function occurring during aging. It is the result of multiple factors, including changes in the metabolic state or in the neuromuscular system, inflammatory pathway activation, altered turnover of contractile proteins and organelles as well as altered production and tissue responsiveness of trophic factors. In particular changes in hormonal level, including decrease of insulin-like growth factor (IGF-1), contribute to sarcopenic condition. We demonstrated that the overexpression of IGF-1 in skeletal muscle was able to counter the decrease in muscle mass, CSA and strength, in 24 months age old mice. Moreover, overexpression of IGF-1 improved insulin sensitivity and glucose tolerance..

**P.05 PGC-1 $\alpha$  overexpression in the skeletal muscle: effects on myogenesis**

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Peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) is a master regulator of mitochondrial biogenesis. In skeletal muscle, PGC-1 $\alpha$  expression is induced by exercise. Along this line, transgenic MCK-PGC-1 $\alpha$ , which overexpress this transcription factor specifically in the skeletal muscle, are characterized by enhanced exercise performance in comparison with wild-type animals<sup>1</sup>; this is mainly due to increased myofiber mitochondrial content that results in markedly improved energy metabolism. In addition to an increased proportion of oxidative fibers vs glycolytic ones<sup>2</sup>, we found a high number of fibers with centrally located nuclei, which is indicative of muscle regeneration. Moreover, myogenic stem cells are more abundant in transgenic mice compared to wild-type animals, and when isolated and cultured in differentiating medium, they form larger myotubes. Starting from this point, the aim of the study was to investigate if stem cells from MCK-PGC-1 $\alpha$  mice can improve myogenesis. Muscles from male wild-type and MCK-PGC-1 $\alpha$  mice were subjected to mild digestion and mononuclear cells were isolated by filtration. These cells were then transplanted into the *tibialis anterior* muscle of female wild-type mice, either injured (BaCl<sub>2</sub> i.m. injection 8 hours before cell transplantation) or not. The animals were euthanized 12 days after BaCl<sub>2</sub> injection. Hematoxylin/eosin staining of muscles transplanted with WT-derived cells shows improved regeneration. On the contrary, all the muscles injected with MCK-PGC-1 $\alpha$ -derived cells show an increase of centrally located nuclei, altered myofiber cross-sectional area distribution and marked SDH staining. The expression of molecular markers of regeneration is consistent with the histological pattern.

The results obtained in the present study suggest that cells isolated from MCK-PGC-1 $\alpha$  donor mice are able to fuse with recipient muscle myofibers partially inducing a shift towards oxidative metabolism and affecting regeneration.

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**P.06 In early stages of cancer-cachexia, loss of mass of fast-twitch fibers results from mitochondria and SR reduction.**

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Fast-twitch fibers are more affected during the muscle waste induced by cancer cachexia. The current literature correlates the proteolysis-related atrophy (which usually occurs in later stages of cachexia syndrome) to oxidative damage of proteins. In the present study, we investigated oxidative modifications, ultrastructural modifications in mitochondria and sarcoplasmic reticulum (SR), and the correlation with muscle loss in EDL and Soleus muscles (respectively fast- and slow-twitch muscles) in an early stage of cachexia (pre-cachexia). Male Wistar rats were subcutaneously inoculated with a suspension of Walker-256 tumor cells, and divided in 2 groups: tumor bearing rats (T), and tumor bearing rats treated with N-acetylcysteine (T-NAC; NAC 1% *ad libitum* in drinking water), a drug known to promote increased antioxidant capacity in tissues. A control group (C) without tumor implantation or NAC treatment was also added. After 5 days, pre-cachexia was characterized in T animals according to the criteria pre-established by Fearon et al. (2011; *Lancet Oncol*). Tumor implantation caused decrease in general body weight (-2.77%), followed by a significant weight loss in EDL muscle (-15.33%), contrasting with the small mass loss of soleus (-5.14%). We measured both protein and membrane oxidation and found that only membrane oxidation was significantly increased in EDL from tumor bearing mice. As a result, we found that the volume of intracellular membrane organelles such as SR and mitochondria was significantly decreased (-18.89% and -22.41%, respectively) compared to group C. Interestingly, all differences were significantly rescued in T-NAC group, suggesting a central role of oxidative stress in the modifications of membrane compartments. These data suggests that in pre-cachectic stages: 1) glycolytic muscles are already more prone to mass waste, but independently of protein oxidation; and 2) loss of membrane organelles could play an important role early in these initial stages.

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**P.07 Caveolin-3, MURC and Trim72 predict myogenic differentiation in the human embryonal rhabdomyosarcoma RD cell line**

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Caveolin-3 (Cav-3), muscle-restricted coiled coil MURC (also referred to as Cavin-4) and the recently identified large tripartite motif Trim72 are proteins controlling a number of important processes in skeletal muscle, ranging from myogenesis and insulin signaling up to sarcolemma repair. In this study we investigated their expression in rhabdomyosarcoma (RMS), a soft tissue tumor showing morphological and biochemical traits of the skeletal muscle lineage. Immunohistochemical analysis showed a similar pattern of expression of the three proteins in different human RMS samples. Immunoblotting analysis carried out using different RMS cell lines showed an increased expression of these proteins during myogenic differentiation as compared to proliferating cells. In addition, confocal microscopy analysis revealed that Cav-3, MURC and Trim72 all co-localize at the plasmalemma of differentiated embryonal RD cells. Ectopic expression of a point mutated Cav-3<sup>P104L</sup> form in RD cells was sufficient to mislocalize both MURC and Trim72 into perinuclear Golgi-like compartments, leading to a cell phenotype characterized by increased ERK phosphorylation and severe impairment of myogenic differentiation. Overall, these data suggest that a concurrent expression of Cav-3, MURC and Trim72 configures as a specific signature predicting and regulating cell differentiation in RMS.

**P.08 Physical preconditioning prior to hindlimb unloading preserves gastrocnemius mass**

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It has been shown that mitochondrial dysfunction modulates intracellular pathways involved in skeletal muscle mass control and plays a major role in disuse atrophy. As aerobic exercise can promote an oxidative metabolic program, we hypothesized that exercise training before hindlimb suspension could prevent

mitochondrial dysfunction and skeletal muscle atrophy. To test the latter hypothesis the effects of aerobic physical preconditioning on mechanisms involved in muscle mass maintenance and muscle mass were studied. Mice were divided into 4 groups: 7 days aerobic training before suspension (EX+HU); 7 days exercise only (EX); 3 days of hindlimb unloading (HU); control (CTRL). Mice were trained daily on treadmill for 7 days. After 3 days HU, gastrocnemius showed atrophy (CSA: CTRL 1557±26 vs HU 1300±134), ubiquitine proteasome system (Murf1 and atrogin1 increase), autophagy activation (LC3II/LC3I increase) and no change of synthetic pathway (mTOR) compared to control mice. Gastrocnemius of EX+HU mice did not show muscle mass loss (CSA: HU 1300±134 vs 7EX+HU 1479±5). Such prevention of muscle atrophy in EX+HU was associated with: (i) a significant enhancement of TFAM and NRF-1 mRNA levels, two transcription factors regulating mitochondrial biogenesis; (ii) a significant increase of mRNA levels of key factors involved in mitochondrial dynamic, namely profusion proteins Mfn2, OPA1 and profission proteins DRP1 and Fis1; (iii) an increased phosphorylation of mTOR compared to control and HU mice; (iv) a significant persistent activation of catabolic pathways. The data suggest that exercise training can prevent skeletal muscle atrophy due to acute periods of unloading by modulating mitochondrial dynamics.

**P.09 Exercise role on myotendinous junction modulation: a morphofunctional study**

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The muscle-tendon interface, called myotendinous junction (MTJ), is the key anatomical area through which the contractile strength can be transmitted between tissues. The MTJ is a dynamic interface and different physiological and pathological conditions may induce morphofunctional changes. Mechanical loading seems to have a key role in the MTJ plasticity. In fact, MTJ may reduce or increase its complexity and the contact surface between tissues, in relationship to muscle atrophy and exercise protocols, respectively. The molecular mechanisms and the different stages of these morphofunctional adaptations need to be further deepened. An interesting high presence of tenocytes near the MTJ has been revealed by means of transmission electron microscopy. These cells showed an increased amount of rough endoplasmic reticulum in exercised rats, compared to sedentary ones. In literature, the exercise ability to increase tenocyte rough endoplasmic reticulum amount has already been

demonstrated. This increase has been associated with enhanced production of collagen fibers and extracellular matrix components. Morphological observations of tenocyte behaviour near MTJ reveal their activation following exercise protocol, suggesting a key role of these cells in the modulation of MTJ morphology.

#### **P.10 Electrical pulse-stimulation of myotubes induces extracellular vesicle release**

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Regular exercise has emerged as an effective strategy to prevent and treat metabolic diseases. The beneficial effects of exercise can be easily observed in muscle tissue but also on a variety of distant organs, such as brain, heart, lungs, adipose tissue and liver, thus suggesting that muscle has endocrine activity. A growing body of evidence shows that contracting muscle releases cytokines with autocrine, paracrine and endocrine functions; hence, they are now recognized as myokines. In the last years a new mechanism for intercellular communication mediated by extracellular vesicles (EVs) has emerged. EVs are spherical structures bound by a lipid bilayer, which is similar in composition to the cell membrane from which the vesicle originated. Among these, exosomes are well-defined vesicles (ranging in size from 40 to 100 nm) that originate from multivesicular bodies. In order to understand EV release in working skeletal muscles, we took advantage of electrical pulse stimulated (EPS) C2C12 myotube model to investigate the characteristics of secreted EVs in response to EPS-evoked contractile activity. Five to six days after differentiation, C2C12 myotubes were placed in a chamber for electrical stimulation. EPS was carried out as follows: 6 hours stimulation with continuous 10-ms duration pulse of 40V/60mm voltage and 1 Hz frequency. Conditioned medium was first cleared and then exosomes were collected using standard ultracentrifugation protocol. EPS applied for 6 hours resulted in contraction of the myotubes and no other dramatic morphological changes were detected. Although EPS promoted the contraction of C2C12 myotubes, it did not appear to induce cell death, as assessed by LDH release, since cytotoxicity did not differ significantly between cell cultures with vs. without EPS treatment. The immunoblotting results showed that phosphorylation of AMP-activated protein kinase (AMPK; Thr172), and ERK1/2 (Thr202/Tyr204) was slightly but significantly increased by 6-h EPS. Then we examined whether EPS-evoked contraction results in cytokine and miRNAs modulation and, as expected, IL-6 mRNA and mir-146a expression were

remarkably upregulated in contractile myotubes. Nanoparticle tracking assay showed that EPS-induced contraction determined an increased release of EVs in the medium compared with control. These data were further corroborated by western blot analysis of purified EVs using antibodies against well-defined exosomes markers (Tsg101 and Alix). Taken together, these findings indicate that myotube contraction may induce the active release of membrane vesicles, in particular exosomes potentially involved in mediating communication between muscle and other organs. Supported by: Italian Ministry of Health: GR-2011-02350264 to MG.

#### **P.11 RAGE and its ligands, S100B and HMGB1, are molecular determinants of cancer-induced muscle wasting**

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Cancer is a recognized cause of the prominently reduced muscle mass known as cachexia. Inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  are main atrophy-inducing factors in cachexia causing excess catabolism of myofibrillary proteins.<sup>1</sup> RAGE (Receptor for Advanced Glycation End-products) and its physiological ligands, S100B and HMGB1, are involved in muscle regeneration, inflammation, and tumor growth, which represent key processes in cancer cachexia.<sup>2,3</sup> We found that: i) RAGE signaling has a trophic effect in myotubes in physiological conditions; ii) excess RAGE ligands leads to myotube atrophy; iii) high amounts of S100B and HMGB1 are found in cachectic muscles, and elevated levels of S100B are present in the serum of cachectic mice, likely released by tumor cells; iv) atrophying muscles re-express RAGE; v) RAGE, S100B and HMGB1 are involved in the mechanism through which TNF $\alpha$ ±IFN $\gamma$  induces atrophy in myotubes *in vitro* and in muscles *in vivo*; vi) Lewis lung carcinoma (LLC)-bearing RAGE-null (*Ager*<sup>-/-</sup>) mice show reduced loss of muscle mass and reduced atrogenes expression, and a dramatic increase in survival rate compared with LLC-bearing WT mice, likely due to reduced systemic inflammation, maintenance of spleen morphology and a different tumor-derived cytokine profile. Thus, increased expression/activity of RAGE and its ligands, S100B and HMGB1 at both systemic and muscle levels appears to concur to muscle wasting in cancer conditions. References: 1. Porporato P.E., 2016, *Oncogenesis*. 22(5): e200; 2. Riuzzi F. et al., 2012, *J. Cell Sci*. 125:1440-54; 3. Riuzzi F. et al., 2012, *PLoS ONE* 7(1): e28700.

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**P.12 Effect of flywheel-based isoinertial exercise on markers of skeletal muscle adaptations**

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Flywheel-based isoinertial exercise was originally designed to maintain muscle health of astronauts during spaceflight. It employs isoinertial technology rather than gravity dependent weights, which allows for maximal concentric and eccentric muscle actions, with brief episodes of eccentric overload. Isoinertial exercise produces greater muscle hypertrophy and peripheral neural adaptations than weight-loaded resistance exercise in healthy subjects probably due to the eccentric overload. The purpose of this study was to analyse the modulation of circulating and local molecular markers of muscle damage and growth/repair over 48h after an isoinertial exercise. Eight male collegiate students (with at least 2 years of squatting experience) performed a session of isoinertial squat exercise (5 sets of 10 maximal reps; 3' rest in-between) on the D11 flywheel device (Desmotec, Italy). Circulating markers analyzed were: muscle creatine kinase (CKM), insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6); measured before and 2, 24, 48h post-exercise. In addition, miRNAs (miR-1, -133b, -206, -146a, -126 and -423) encapsulated in circulating exosomes were quantified before and 2h after exercise. Local markers investigated were: mRNA levels of genes involved in myogenesis and cell cycle control such as IGF-1 isoforms (IGF-1Ea, IGF-1Eb, and IGF-1Ec), myogenin, myogenic regulator factor-4 (MRF-4) and cyclin D1; determined in *vastus lateralis* muscle using fine needle aspiration coupled with real-time PCR before and 2h post-exercise. Exercise-induced inflammatory response was also analyzed by quantification of mRNA encoding cytokines and chemokines (I $\kappa$ B- $\alpha$ , MCP-1, TNF $\alpha$ , IL-6 and IL-6R) in muscle and in peripheral blood mononuclear cells (PBMC). Circulating CKM increased significantly after 2h post-exercise, the maximum peak occurred within 24h restoring to baseline level within 48h. Isoinertial exercise significantly increased total serum IGF-1 24h post-exercise and IL-6 at 2, 24 and 48h post-exercise. Nanoparticle tracking assay revealed a 2-fold increase in circulating exosomes in response to acute exercise, which was paralleled by higher levels of the mir-146a and mir-126. Exercise increased muscle MCP-1, TNF- $\alpha$

and IL-6 and peripheral PBMC I $\kappa$ B- $\alpha$  and MCP-1 mRNA levels 2h post-exercise. On the contrary, muscle IGF-1Ea, IGF-1Eb, IGF-1Ec myogenin and cyclin D1 mRNA content was down-regulated 2h after the exercise bout. Muscle I $\kappa$ B- $\alpha$ , IL-6R and MRF-4 and PBMC TNF- $\alpha$ , IL-6 and IL-6R mRNA levels were unaffected. In conclusion, a single isoinertial exercise session increased serum markers of muscle damage (CKM and IL-6) and pro-inflammatory gene expression in muscle and PBMC of healthy, recreationally resistance-trained men. On the contrary, the mRNA level of genes related to muscle growth and repair decreased 2h post-exercise, suggesting that these processes had not yet been activated while those responding to muscular injury were prevalent. Accordingly, the plasma levels of IGF-1 increased only after 24 h post-exercise. These results indicate early molecular adaptations of skeletal muscle to loading, supporting the hypothesis that eccentric-overload inferred by isoinertial machinery offers a potent stimulus to critically optimize the benefits of resistance exercise.

**P.13 Unraveling muscle slowness in NEM6 myopathy: a key role for the skeletal muscle thin filament**

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Nemaline myopathy (NM) is among the most common non-dystrophic congenital myopathies. Recently, a novel implicated gene was discovered - *KBTBD13*. NM patients with mutations in *KBTBD13* (NEM6) exhibit muscle weakness and a typical muscle slowness. Here, we aim to gain insight in the pathophysiology of NEM6 myopathy muscle slowness. *In vivo* muscle relaxation was assessed using Transcranial Magnetic Stimulation (TMS) in NEM6 patients (n=10) and controls (CTRL) (N=24). Calcium-handling protein levels were determined in skeletal muscle biopsies by Western blot. Contractile parameters were measured in isolated single fibers and in myofibrils that were isolated from skeletal

muscle biopsies. Next, the nanoscale structure and acto-myosin interactions in these muscle fibers were studied by X-ray diffraction. *In vivo* TMS revealed slower muscle relaxation in NEM6 patients. The phosphorylated phospholamban/phospholamban ratio was lower in NEM6 muscle biopsies, which might contribute to slower calcium-uptake. Relaxation kinetics of both single muscle fibers as well as individual myofibrils were slower in NEM6 compared to CTRL. X-ray diffraction studies show that the peak position of the actin layer line 6 was reduced in NEM6 compared to CTRL, suggesting a compressed, stiffer thin filament. Modelling of sarcomere kinetics revealed that a stiffer thin filament slows muscle relaxation. Here, we studied the pathophysiology of muscle slowness in NEM6 patients. We used a top-to-bottom approach: from the patient *in vivo* level to the nanoscale acto-myosin *in vitro* level. The data suggest that changes in the skeletal muscle thin filament level contribute to the clinical phenotype of NEM6.

#### P.14 Zinc finger E-box-binding homeobox 2 a new player in skeletal muscle differentiation

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Myogenic helix-loop-helix proteins such as MyoD, Myf5, myogenin and MRF-4 regulate the skeletal muscle development and differentiation. In addition, a few zinc finger proteins have been described as helix-loop-helix proteins and are also regulators of muscle development and muscle gene expression. Among those proteins, Zinc finger E-box-binding homeobox 2 (Zeb2, also known as SMAD interacting protein 1) is poorly characterized although showing similar molecular characteristic of skeletal muscle regulators (1). In this study we investigate whether Zeb2 could play a role in regulating myogenic differentiation using genetic tools and transgenic mouse embryonic stem (mES) cells. We report that mES cells genetically modified for the absence or the overexpression of Zeb2 maintain pluripotency and are capable to differentiate in skeletal muscle cells. However, Zeb2-null mES cells display impaired myogenic differentiation and more

interestingly the overexpression of Zeb2 in both mES cells and adult myogenic cells impacts positively the myogenic commitment *in vitro* and *in vivo*. To deeper investigate the role of Zeb2 in myogenic differentiation and in the context of cellular microenvironment we employed the single cell sequencing (RNA-Seq) in transgenic mES cells. This technology examines the sequence information from individual cells with optimized next generation sequencing technologies (2). The single cell RNA-Seq analysis shows that myogenic regulatory factors including Id family and SMAD/TGF- $\beta$  pathway genes are affected by the absence or the overexpression of Zeb2. These findings reveal a critical role of Zeb2 in skeletal muscle differentiation, thus providing a novel possible target to improve skeletal muscle regeneration in muscle diseases.

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#### P.15 Physiopathological characterization of the role of MCUB in skeletal muscle regeneration

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Mitochondrial calcium uptake plays a key role in modulating cell metabolism, cell survival and other cell specific functions. Calcium accumulates into the mitochondrial matrix through the mitochondrial calcium uniporter (MCU). Few years ago a MCU homolog has been discovered, which has been called MCUB. MCU and MCUB shares 50% sequence and structure similarity although some conserved differences in the primary sequence prevent MCUB from forming a Ca<sup>2+</sup>-permeable channel, thus acting as a dominant-negative subunit. RT-PCR experiments demonstrated that MCUB expression levels dramatically increase during skeletal muscle regeneration after cardiotoxin-induced injury. In addition, high MCUB expression levels have been detected in anti-inflammatory macrophages (M2). The latter are one of the most important effectors of the later stages of tissue repair. Preliminary results demonstrated that MCUB has a key role in macrophages skewing from M1 to M2 phenotype. In order to confirm our hypothesis, we performed skeletal muscle regeneration experiments on a MCUB KO mouse model. Our preliminary results demonstrate that the lack of MCUB causes a delay in skeletal muscle regeneration process that occurs in parallel with a reduction of the expression level of known markers of M2. We hypothesized that this altered muscular phenotype might be due to an

impairment in macrophages skewing from an M1 to M2 phenotype. Indeed, macrophages from MCUb KO animals have lower phagocytic capacity compared to wild type animals and this affects skeletal muscle regeneration. These results are in line with published data demonstrating that phagocytic activity is fundamental for M2 polarization.

**P.16 mIGF-1 over-expression ameliorates dystrophic muscle niche improving the efficacy of DMD therapies in mdx mice**

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Duchenne muscular dystrophy (DMD) is a pathological condition caused by the absence of a functional dystrophin protein. To date, there is no effective therapy for DMD; however, alternative therapeutic approaches have been attempted. Cell-based therapies are promising methods for treating DMD but stalled by a limited impact of transplanted stem cells on the long-term muscle cell replacement. Gene therapy, including exon-skipping, gained interest because of optimistic results in clinical trials but antisense oligonucleotides (AO) needs to be chronically injected to maintain a clinical efficacy. Our working hypothesis is that the hostile dystrophic microenvironment might interfere with and limit the efficacy of DMD therapies. A potential candidate that contributes to sustain a more hospitable microenvironment in dystrophic muscle is the Insulin-like growth factor-1 (IGF-1). We previously demonstrated that local IGF-1 over-expression plays a critical role in muscle regeneration modulating muscle-specific genes associated with maturation of regenerating fibers. Thus, mIGF-1 over-expression in dystrophic context could be useful for cell-based therapy, sustaining resident and transplanted stem cells survival and could improve the efficacy of the exon skipping-mediated approach through the stabilization of the differentiated phenotype in mdx muscle. In this work we show how mIGF-1 over-expression in mdx mice is able to modulate pathological mechanisms affecting muscle niche. Moreover, we analysed the impact of the modulation of dystrophic environment by mIGF-1 on both stem cell therapy, based on mesoangioblasts transplantation, and gene therapy performed using PMO-Pip6a, a new generation PMO-based AO conjugated to the Pip6a peptide enhancing the delivery in mdx mice.

**P.17 The HDAC inhibitor givinostat counters the atrophy program induced by TNF- $\alpha$  in human skeletal myotubes**

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Histone deacetylase (HDAC) and histone acetyltransferase (HAT) catalyse the reactions that maintain the homeostatic level of lysine side chain acetylation in thousands of cellular proteins. In muscle cells, alteration of this equilibrium is found in a variety of pathological conditions characterized by progressive degeneration and atrophy. Muscle atrophy may occur as a consequence of genetic diseases such as Duchenne muscular dystrophy (DMD). DMD is caused by mutations in the dystrophin gene that lead to the absence of the functional protein, myofiber membrane instability and damage upon contraction. Consequently, muscle cells release cytoplasmic components including the so-called Damage Associated Molecular Patterns (DAMPs). These activate both the resident immune cells and the muscle cells thus generating an inflammatory environment that progressively increases and amplifies the muscle damage. NF- $\kappa$ B activation is one of the downstream effect of TLR activation through DAMPs and proinflammatory cytokines such as TNF- $\alpha$ . These pathways are subjected to regulation involving the acetylation of effector molecules. HDAC inhibitors are endowed with pleiotropic activities that influence the biology of muscle cells at different levels. Givinostat is a pan-HDAC inhibitor currently in phase III clinical trial for the treatment of DMD. Immunohistochemistry analysis of patients' biopsies demonstrated that treatment with givinostat lead to a reduced inflammation, necrosis, fibrosis and increase of the cross sectional area of the myofibers that occupy a larger fraction of muscle tissue. To further characterize the molecular mechanism of action of givinostat on muscle cells induced atrophy we used in vitro differentiated human skeletal myotubes stimulated with the atrophy inducer cytokine TNF- $\alpha$ . We found that givinostat countered the upregulation of some key atrogenes such as TRIM63 and Atrogin-1 induced by TNF- $\alpha$ . Muscle cells treated with TNF- $\alpha$  activated the expression of HDAC4 and HDAC6, both involved in muscle atrophy. Furthermore, HDAC6 has been recently described as an atrogene that interact with Atrogin-1 multiprotein complex. As expected, givinostat induced tubulin hyperacetylation, a pharmacodynamic marker of HDAC6 inhibition. Since givinostat inhibits HDAC4 activity, both enzymes are dually inhibited, at transcriptional and functional level. In agreement with these gene modulations, we found that givinostat upregulated the gene expression of myosin heavy chain I and II that are targeted to proteasomal degradation

during atrophy. Taken together, these data indicate that givinostat counters the atrophy program induced by TNF- $\alpha$  in normal skeletal muscle cells and suggest that a similar mechanism may take place in DMD muscle cells.

#### **P.18 Investigating the cell origin and heterogeneity of embryonal Rhabdomyosarcoma**

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With an incidence of 4.5 cases per million adolescents, the rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma. It develops in different tissues, most commonly in the head and neck, in the extremities and in the genitourinary tract. According to its histological and pathological characteristics, RMS can be classified in two major subtypes, embryonal (eRMS) and alveolar (aRMS), which seem to share the same initiating cell type(s), even if this point is still debated. In fact, some evidence supports the notion that skeletal muscle progenitors, such as satellite cells, could give rise to RMS even though alternative theories point to mesenchymal stem cells or even progenitors of the adipocyte lineage, as possible tumor-initiating cells. The clinical differences between the two RMS types result from different molecular genetic mechanisms of origin. To study the eRMS, which is our main focus, we adopted the KrasG12D/+Trp53Fl/Fl conditional mouse model to induce cell transformation, by in vivo or in vitro infecting cells with an Adenovirus vector expressing the CRE recombinase that leads to the constitutive activation of the oncogene KRAS along with the inactivation of the P53 tumor suppressor gene. Since our goal is to identify which cell population(s) can give rise to eRMS, we triggered embryonal RMS formation by infecting purified muscle mononuclear cell populations with the CRE recombinase adenovirus which activates expression of Kras(G12D) and inactivates the p53 gene. Both satellites and FAPS are transformed in vitro by this approach and induce the formation eRMS like tumors when grafted into nude mice. We are in the process of characterizing the changes in the cell populations from the tumor mass, at different stages of development, by flow cytometry techniques.

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#### **P.19 Amotl2 and Homer1 – novel regulators of the postsynaptic machinery organization**

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Mammalian neuromuscular junctions (NMJs) undergo a postnatal topological transformation from a simple oval plaque to a complex branch-shaped structure called “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, assemblies formed in vitro undergo similar stages in developmental remodeling from “plaques” to “pretzels” as the NMJ postsynaptic machinery 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 scaffolding protein Amotl2, which is localized at podosomes. We now report that in muscle cells Amotl2 interacts with one of the key synaptic components, Homer1. Mice lacking Homer1 expression exhibit skeletal muscle myopathy characterized by disturbed calcium homeostasis. Additionally, it has been shown that Homer1 expression is affected in mouse models of Amyotrophic Lateral Sclerosis (ALS) and Duchenne’s Muscular Dystrophy (DMD). We show that Homer1, together with Amotl2 are concentrated at postsynaptic areas of NMJs in the indentations between the acetylcholine receptor-rich branches. We also demonstrate that Homer1 is indispensable for AChR clustering in the cultured myotubes and identify Homer1-interacting proteins that are involved in this process. Our results provide novel insight into molecular machinery that is orchestrating postsynaptic machinery development.

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#### **P.20 Biochemical investigations to unravel myopathological perturbations caused by the Caveolin-3 p.P104L mutation**

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Caveolin-3 (CAV3) is a muscle specific protein localized to the sarcolemma where it interacts with the dystroglycan complex (DGC) and is thus involved in the connection between the extracellular matrix (ECM) and the cytoskeleton. Muscle diseases caused by mutations in the CAV3 gene are called Caveolinopathies. So far, more than 40 dominant pathogenic mutations have been described leading to different phenotypes molecularly associated with a mis-localization of the mutant protein to the Golgi. Hereby, associated Endoplasmic Reticulum (ER)-stress has been demonstrated for the p.P104L mutation. However, the further pathophysiological consequences of mutant CAV3 mis-localization and ER-stress remained elusive. Utilizing a transgenic (p.P104L) mouse model of Caveolinopathy and performing proteomic profiling along with immunoblot and morphological studies (including electron and CARS microscopy) we systematically addressed these consequences. Our morphological studies revealed Golgi and ER proliferations as well as the build-up of protein aggregates. These observations were confirmed via immunological studies and are in accordance with our proteomic data showing altered abundance of 120 proteins in diseased quadriceps muscle fibres. Proteomic findings indicated ECM remodeling and cytoskeletal vulnerability. Moreover, our proteomic findings suggested that further DGC components are affected by the perturbed protein processing machinery leading to the formation of protein aggregates which could be confirmed via CARS microscopy. Hence, our combined data classify (p.P104L) Caveolinopathy as an acquired protein folding disease with sarcolemmal affection and thus expand the pathophysiological knowledge of this disorder, an important aspect in the therapeutic management of CAV3-patients.

#### **P.21 Extracellular vesicles released during myogenic differentiation trigger changes in macrophage phenotype**

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Skeletal muscle is a highly plastic tissue able to adapt to different stresses, in part due to its remarkable regenerative capacity. Recently, it has been found that myoblasts and myotubes release exosomes and exosome-like vesicles in the extracellular environment during myogenic differentiation. Within the satellite cell niche, muscle stem cells exchange signals with other cell types, and among these, complex interactions between skeletal muscle and the immune system have been reported. The aim of this study was to ask whether EVs (Extracellular Vesicles) released by differentiating myocytes can mediate cell-communication between muscle cells and macrophages, one of the key actors of muscle remodelling. To this end, RAW 264.7 cells (a model of macrophages) have been used as target cells. Myocytes undergoing myogenic differentiation are subjected to deep structural rearrangements, so that a complex mixture of EVs (comprising exosomes and shedding microvesicles) and cellular membrane fragments may be released in the extracellular environment. A serial ultracentrifugation protocol was specifically adjusted to remove cellular debris and isolate shedding microvesicles and exosomes. The EVs collected during myogenic differentiation process were characterized using TEM, western blot, density gradient and real-time PCR analyses and used to treat RAW 264.7 cells. Gene expression analysis performed 24 h after treatment highlighted a significant up-regulation of IL-6 and IL-10, two cytokines involved in muscle differentiation and anti-inflammatory process, respectively, but not of TNF-alpha (a proinflammatory cytokine). IL-6 stimulates the production of the classical anti-inflammatory cytokines IL-1Ra and IL-10, moreover it has been reported that IL-10 plays a central role in regulating the switch of muscle macrophages from a M1 to M2 phenotype in injured muscle in vivo. Collectively, these data suggest that EVs could be involved in the regulation of normal growth and regeneration of muscle.

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#### **P.22 Pericyte in the muscle satellite cell niche: a key player maintaining the steady state and helping recovery.**

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Muscle growth and regeneration following injury, is regulated by the myogenic stem cells, called satellite cells. The satellite cells are located beneath myofiber

basement membranes and closely associated with capillary endothelial cells. We previously observed that 90% of capillaries were associated with pericytes in adult mouse and human muscle. We also have shown during post-natal growth that, by promoting post-natal myogenesis through Insulin like-growth factor 1 and stem cell quiescence through Angiopoietin-1, pericytes play a key role in the microvascular niche of satellite cells. Consistently, here we show that in a mouse model of muscle pericytes depletion, the loss of the perivascular cells induces a spontaneous necrosis of the muscle. We also observed after induced chemical injury of the muscle in a mouse model of depletion of microvascular Angiopoietin-1, a delayed regeneration process associated with Type 2 myofibers hypotrophy along with an elevated number of remaining cycling Pax7+ cells. In conclusion, pericytes associated with endothelial cells are essential to maintain adult muscle homeostasis and exert paracrine effects on adjacent myogenic cells during muscle repair in adulthood.

### **P.23 Silencing Nfix rescues Muscular Dystrophy by delaying muscle regeneration**

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Muscular Dystrophies are severe disorders due to mutations in structural genes that cause skeletal muscle wasting compromising patient mobility and respiratory functions. Although previous works suggested enhancing regeneration and muscle mass as therapeutic strategies, these led to no long-term benefits in humans. Here we propose a conceptually new idea based on making a dystrophic muscle slower in regeneration and more oxidative, by silencing of the transcription factor *Nfix*. In different forms of Muscular Dystrophy, lack of *Nfix* rescues histopathological and functional hallmarks of dystrophic muscle. More importantly, silencing *Nfix* in post-natal dystrophic mice, when the first signs of the disease already occurred, rescues the pathology. On the contrary, *Nfix* overexpression in dystrophic muscles pushes regeneration and markedly exacerbates the pathology. We therefore provide evidence that current strategies are based on a misconception and offer a proof of principle for a novel therapeutic approach.

### **P.24 Crosstalk between oxidative stress and inflammation in dystrophic muscle: focus on IL6 signaling**

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Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations in the dystrophin gene. Oxidative stress and chronic inflammation have been proposed as important mechanisms involved in DMD. Under pathological conditions, elevated levels of ROS in skeletal muscle can overwhelm cellular antioxidant defenses, leading to oxidant-related damage. Moreover, an alteration in the redox signaling might stimulate inflammatory response collaborating to produce muscle fiber necrosis. To date, inflammation is considered the principal determinant of degenerative processes in dystrophic muscle and treatments are limited to glucocorticoids. However, a central role of oxidative stress in DMD pathology has been evidenced by clinical and pre-clinical studies. A potential candidate linking inflammation and ROS production is Interleukine 6 (IL6), a critical factor involved in the switch between acute and chronic inflammation. We generated a severe animal model of DMD, the mdx/IL6 mouse, that better recapitulates disease progression in human pathology and approximates the antioxidant expression profile observed in DMD patients. In particular, increased circulating levels of IL6 alter the redox signaling cascade in dystrophic muscle.

### **P.25 Caveolin-1 overexpression accelerates tumor growth and metastasis of embryonal rhabdomyosarcoma**

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Caveolin-1 (Cav-1) is a plasma membrane scaffolding protein that was shown to control the ERK pathway in muscle satellite cells. Oncogenic transformation of satellite cells is responsible of the generation of rhabdomyosarcoma (RMS), a soft tissue tumor affecting childhood and adolescence. We previously reported that Cav-1 is a marker of proliferating RMS cell lines and that its overexpression promotes increased malignancy of RMS cells in vitro and in vivo. Here we show that tail vein injection of the human embryonal RD cells with Cav-1 overexpression (RD<sup>Cav-1</sup>) into NOD/SCID mice resulted in formation of lung metastasis in about 9 weeks as compared to control cells that did not form

metastasis. After performing *ex vivo* transplantation of lung metastases we isolated one cell population, termed lung metastatic RD1, which injected in mice again gave rise to lung metastases in 5 weeks; from these disseminated lungs we were able to isolate the lung metastatic RD2 cell population. All the distinct cell populations, including RD<sup>Cav-1</sup> and lung metastatic RD1 and RD2 clones, retained high Cav-1 expression and showed high phosphorylation levels of ERK1/2, which completely prevented their ability to undergo myogenic differentiation. In addition, lung metastatic RD1 and RD2 clones exhibited an increased migration, adhesion and production of angiogenic stimuli in comparison to non-metastatic control RD and RD<sup>Cav-1</sup> lines. Taken together, these data suggest a key role of Cav-1 in promoting both local tumor growth and metastasis of RMS through cooperation of the ERK signaling pathway.

#### **P.26 Oxidative stress in Duchenne muscular dystrophy: focus on the NRF2 redox pathway**

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Oxidative stress is involved in the pathogenesis of Duchenne muscular dystrophy (DMD), an X-linked genetic disorder caused by mutations in the dystrophin gene and characterized by progressive, lethal muscle degeneration and chronic inflammation. In this study, we explored the expression and signaling pathway of a master player of the anti-oxidant and anti-inflammatory response, namely NF-E2-related Factor 2, in muscle biopsies of DMD patients. We classified DMD patients in two age groups (Class I, 0–2 years and Class II, 2–9 years), in order to evaluate the antioxidant pathway expression during the disease progression. We observed that altered enzymatic antioxidant responses, increased levels of oxidized glutathione and oxidative damage are differently modulated in the two age classes of patients and well correlate with the severity of pathology. Interestingly, we also observed a modulation of relevant markers of the inflammatory response, such as heme oxygenase 1 and Interleukin-6 (IL-6), suggesting a link between oxidative stress and chronic inflammatory

response. Of note, using a transgenic mouse model, we demonstrated that IL-6 overexpression parallels the antioxidant expression profile and the severity of dystrophic muscle observed in DMD patients. This study advances our understanding of the pathogenic mechanisms underlying DMD and defines the critical role of oxidative stress on muscle wasting with clear implications for disease pathogenesis and therapy in human.

#### **P.27 Role of ghrelin peptides in aging**

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Sarcopenia is a multifactorial syndrome defined as the irreversible loss of skeletal muscle mass and functionality occurring during aging. Muscle atrophy and impaired regeneration are the main features of this syndrome but the underlying mechanisms and etiology remain poorly defined. Acylated and unacylated ghrelin (AG and UnAG, respectively) are circulating peptide hormones mainly produced in the stomach. AG, through GHSR-1a, induces a strong release of GH and orexigenic effects. Although UnAG does not activate this receptor, shares common activities with AG on skeletal muscle counteracting atrophy and promoting myoblast differentiation. Moreover, UnAG enhances muscle regeneration stimulating satellite cell functions. AG/UnAG plasmatic levels change during aging and this may contribute to sarcopenia establishment. We are evaluating the role of AG/UnAG in sarcopenia prevention by means of Myh6/Ghrl transgenic (Tg) mice (characterized by high levels of circulating UnAG) and of *Ghrl*<sup>-/-</sup> (KO) mice. Although no difference in muscle functionality were observed among Tg, KO and WT at 6 and 12 months, fat accumulation and glucose clearance rate were significantly different in the different genotypes. Moreover, muscle regeneration in KO mice was impaired, suggesting a potential role of AG/UnAG system in the sarcopenia onset.

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#### **P.28 circZNF609: a circular RNA involved in myoblast proliferation and in rhabdomyosarcoma**

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Circular RNAs represent a recently re-discovered class of covalently closed RNAs, derived from a non-canonical splicing event (back-splicing), ubiquitously expressed among Eukaryotes and conserved among different species. We identified several circular RNAs expressed during myogenesis. Thanks to a knockdown-based phenotypic screening, we identified a circRNA, named circZNF609, involved in the regulation of human myoblast proliferation. Upon its depletion, the percentage of proliferating cells is highly reduced with respect to the control sample. CircZNF609 also has an open reading frame generated upon circularization and it can encode a peptide. Here we focus on circZNF609 role in regulating cell cycle progression. An RNAseq experiment performed on human myoblasts revealed that the expression of 300 genes is altered upon circZNF609 specific depletion, of which 60% are down-regulated, and specifically enriched for cell cycle related genes. To deepen our knowledge about circZNF609 role in proliferation, we studied its expression and function in rhabdomyosarcoma (RMS), a pediatric muscle malignancy. We found that circZNF609 is strongly up-regulated in biopsies from the two major RMS subtypes, the embryonal and the alveolar, and we discovered that its knockdown blocks proliferation of an RMS-derived cell line, promoting an accumulation of cells in G1 cell cycle phase, with a reduction of cells in S phase. These results suggest that circZNF609 could be a good target for therapeutic approaches against RMS. We are now testing the effect of its depletion on tumor growth in xenograft mice, injected with a stable RMS cell line expressing an siRNA against circZNF609 upon doxycycline induction.

**P.29 Sertoli cells protect C2C12 myotubes against atrophy and induce utrophin expression in canine and human dystrophic myotubes**

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Sertoli cells (SeC), which are crucial for germinal cell development, have demonstrated trophic and

immunomodulatory effects in numerous experimental setting.<sup>1</sup> A single injection of microencapsulated SeC into the peritoneal cavity of *mdx* mice, an experimental model of Duchenne muscular dystrophy (DMD), results into recovery of muscle architecture and performance in the absence of any pharmacological immunosuppression, opening to new perspectives for DMD treatment.<sup>2</sup> Besides restraining muscle inflammation, treatment of *mdx* mice with microencapsulated SeC induced at the sarcolemma of myofibers the expression of the dystrophin paralogue, utrophin, thus conferring an additional advantage to dystrophic muscles.<sup>2</sup> However, the direct effects of SeC on myoblasts/myotubes themselves and on myotubes of higher mammals have not been investigated yet. C2C12 myotubes treated with TNF $\alpha$ /IFN $\gamma$  or cultured in PBS (phosphate buffered saline) to mimic atrophy conditions show reduced levels of myosin heavy chain (MyHC), and we found that SeC protect against loss of MyHC in both conditions in a dose-dependent manner, high numbers of SeC being the most efficacious. Moreover, we demonstrated that, as for *mdx* myotubes/myofibers,<sup>2</sup> SeC are able to induce utrophin expression in myotubes from GRMD (golden retriever muscular dystrophy) dogs and dystrophic humans. Our data further support the use of microencapsulated SeC for treatment of DMD patients.

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**P.30 Creatine preserves the myogenic capacity of oxidant-stressed C2C12 myoblasts through mitochondrially-targeted mechanisms**

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Creatine (Cr) is a nutritional supplement promoting a number of health benefits, whose use is spreading in the prevention of muscle aging and treatment of neuromuscular maladies (Wallimann, 2007). Indeed Cr has been shown to be beneficial in disease-induced muscle atrophy, improve rehabilitation and afford mild antioxidant activity (Tarnopolsky, 2011). The beneficial effects of its supplementation are likely to derive from pleiotropic interactions. In accord with this notion, we previously demonstrated that multiple, pleiotropic effects account for the capacity of Cr to prevent the differentiation arrest caused by oxidative stress in C2C12 myoblasts, namely: increased expression of

muscle regulatory factors mRNA, antioxidant activity, amelioration of energy status and preservation of mitochondrial damage (Sestili, 2015). Given the importance of mitochondria in supporting the myogenic process, here we further explored morphologically, cytofluorimetrically and biochemically the protective effects of Cr on the structure, function and networking of these organelles in C2C12 cells differentiating under oxidative stressing conditions (acute exposure to 0.3 mM H<sub>2</sub>O<sub>2</sub>); the effects on the energy sensor AMPK, on *PGC-1 $\alpha$* , which is involved in mitochondrial biogenesis and its downstream effector *Tfam* were also investigated. Our results indicate that damage to mitochondria is crucial in the differentiation imbalance caused by oxidative stress and that the Cr-prevention of these injuries is invariably associated with the recovery of the normal myogenic capacity. We also found that Cr- activates AMPK and induces an up-regulation of *PGC-1 $\alpha$*  expression, two events which are likely to contribute to the protection of mitochondrial quality and function. *References: Sestili et al. (2015) Mini Rev Med Chem. 16, 4-11. Tarnopolsky (2011) Amino Acids 40, 1397-1407. Wallimann (2007) Sub-cell Biochem. 46, 1-16.*

### P. 31 Epigenetic and transcriptomic profiling of primary muscle cells during DMD progression and HDACi treatment

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Duchenne Muscular Dystrophy (DMD) is a lethal X-linked disease caused by mutations in the dystrophin gene that progressively lead to severe respiratory and cardiac failure that cause premature death. DMD pathogenesis is characterized by continuous cycles of muscle contraction/degeneration. At early stages of disease, muscle degeneration is counterbalanced by a compensatory repair driven by muscular stem cells (MuSCs). As the disease progresses, the regeneration potential is exhausted and muscles are replaced by fibrotic scars and fat infiltration (Dalkilic & Kunkel, 2003). The identity of the cellular source of fibrosis and fat deposition has been recently assigned to fibro-adipogenic progenitors (FAPs; Joe et al., 2010; Uezumi et al., 2010). Although there is currently no available cure for DMD, several therapeutic strategies are undergoing investigation. In this context, it is of particular interest the pharmacological approach based on the histone deacetylase inhibitors (HDACi), which represent the first generation of epigenetic drugs used to counteract the DMD progression. Recently, our lab has

identified a regulatory network targeted by HDACi to repress FAPs differentiation into pro-fibrotic and adipogenic cells, while enhancing their ability to support MuSCs-mediated compensatory regeneration (Saccone et al., 2014). Interestingly HDACi pharmacological effect is restricted to the early stage of DMD (Mozzetta et al. 2013). The mechanism during the aging of DMD FAPs that confers resistance to HDACi treatment is still unknown. The aim of this project is thus to investigate the epigenetic and transcriptomic changes that leads FAPs stage dependent response to HDACi. By ChipSeq bioinformatic analysis of MDX FAPs histone acetylation we uncovered an indiscriminate histone hyperacetylation at late stages of DMD that is paradoxically reduced by HDACi treatment. This data could represent the first evidence of an HDACi counter-active effect at late stages of DMD. Integrating various histone modification and the transcriptomic profile of FAPs could unveil a possible mechanism behind DMD aging and HDACi efficacy at late stage of DMD. *References: Dalkilic I, Kunkel LM (2003) Muscular dystrophies: genes to pathogenesis. Curr Opin Genet Dev 13: 231–238. Mozzetta C, Consalvi S, Saccone et al., Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice.*

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### P.32 Iron availability and recycling regulates skeletal muscle wasting

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Iron is an essential metal for a plethora of biological reactions in light of an elevated reactivity, which is also the reason of its elevated toxicity when it is not properly compartmentalized. While iron overload is known to cause muscle dysfunction, we still have a limited knowledge of the role played by iron deprivation in the pathogenesis of skeletal muscle atrophy. Since iron is commonly limited in several systemic diseases characterized by muscle atrophy, we speculated that iron availability might directly regulate skeletal muscle mass homeostasis. Consistently, iron deprivation in vitro is sufficient to promote myotubes atrophy as seen as myotubes thickness reduction and atrogenes

expression. Datasets analysis indicated transferrin receptor 1 (the receptor involved in transferrin uptake) as one of the primary targets to be downregulated during muscle atrophy upon various stimuli, while treatment with conditioned media derived from cancer cells or dexamethasone is sufficient to impair transferrin recycling in myotubes, as well as total transferrin receptor protein levels. These data further suggest that iron metabolism might be directly involved in the pathogenesis of muscle wasting. Implications of the effects of iron dysbiosis on mitochondrial metabolism and altered gene expression will be discussed.

### **P.33 MCU expression levels and mitochondria dynamics in skeletal muscle of 70yrs trained seniors**

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Alterations of mitochondrial  $Ca^{2+}$  homeostasis regulated by MCU has been recently shown to affect muscle trophism *in vivo*. Mice lacking MCU exhibit functional abnormalities in conditions that require a rapid increase in the skeletal muscle work load. During aging skeletal muscle undergoes a progressive loss of muscle mass, with decline in specific force and functional impairment. The etiology of this phenomenon is complex and involves the interplay of numerous factors whose underlying mechanisms are currently not fully understood. Physical exercise is known to have beneficial effects on muscle trophism and force production modulating signaling pathways also via intracellular  $Ca^{2+}$  and specific mitochondrial adaptations. To understand the relevance of MCU-dependent mitochondrial  $Ca^{2+}$  uptake in aging and to investigate the effect of physical exercise on MCU

expression and mitochondria dynamics, we analyzed skeletal muscle biopsies from 70yrs old seniors, either sedentary, 9 weeks and lifelong trained in comparison to young subjects. We demonstrate that improved muscle function and structure induced by physical exercise are linked to increased protein levels of MCU. Ultrastructural analyses by Electron Microscopy showed remodeling of mitochondrial apparatus in trained muscles that is consistent with an adaptation to physical exercise, a response likely mediated by an increased expression of mitochondrial fusion protein OPA1. Altogether these results indicate that the physiological effects of exercise on skeletal muscle size and force are associated with changes in mitochondrial-related proteins involved in  $Ca^{2+}$  homeostasis and mitochondrial shape. These findings observed for the first time in aging human skeletal muscle confirm the data obtained in mice and propose MCU and mitochondria related proteins as potential pharmacological targets to counteract age-related muscle loss, promoting healthy aging.

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### **P.34 The role of Raptor in adult skeletal muscle**

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Mammalian target of rapamycin (mTOR) plays a central role in cell growth. mTOR assembles into two distinct multiprotein complexes, namely the rapamycin-sensitive complex mTORC1 and the rapamycin-insensitive complex mTORC2. One of the key members of the mTORC1 complex is a 150kDa protein called Raptor, which has been shown to be able to recruit mTOR substrates S6K1 and 4EBP1 on mTORC1. Mice lacking Raptor only in skeletal muscle from birth show a pronounced myopathy leading to a premature death. However, treating adult mice with the specific mTORC1 inhibitor rapamycin does not lead to a myopathic phenotype, and even improves muscle physiology in aged mice. Here we want to examine the role of Raptor and mTORC1 using a new CreER-inducible transgenic mouse in which we can delete Raptor in muscles of adult mice (Raptor k.o.). Activation of Cre by treatment with tamoxifen leads to a rapid loss of Raptor transcript and protein levels. Also the phosphorylation levels of ribosomal protein S6, a known mTORC1 target, are strongly reduced in Raptor ko mice. One month after Raptor deletion, muscle weight and basic histology are unchanged. A longer deletion of Raptor, however, leads to a myopathic

phenotype with central-core structures and a high number of small and large muscle fibers.

**P.35 Role of bioactive sphingolipids in skeletal muscle cell degeneration: *Caenorhabditis elegans* as model organism**

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The causes of skeletal muscle (SkM) loss in aging and diseases are complex and largely unknown. Intensive research in simple model organisms, such as the nematode *Caenorhabditis elegans*, which offers the prowess of sophisticated genetic approaches and recapitulates aspects of the human pathologies, may contribute in understanding the molecular mechanisms of SkM cell degeneration. *C.elegans* has distinct striated and non-striated muscle systems, the sarcomere contains the same or homolog morphological features described

in mammals and functional age-related changes of *C.elegans* SkM, including the decline of locomotor behavior, are similar to the age-related or disease-induced modifications observed in mammals. Sphingolipids (SLs), essential components of eukaryotic cell membranes and bioactive factors involved in the regulation of a variety of different cellular processes, such as cell growth and differentiation, are also emerging as key regulators of SkM cell biology. In particular, sphingosine 1-phosphate attenuates the muscle damage, protect the muscle fiber from apoptosis and preserve satellite cell viability and renewal (Sassoli et al., 2011; Meacci et al., 2008). Since the principal components of SL biochemical pathways are also described in the nematode, here, we provide evidence of S1P signaling involvement in the maintaining of SkM phenotype.

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