UNIVERSITÀ DEL PIEMONTE ORIENTALE

ANNUAL REPORT
DEPARTMENT OF TRANSLATIONAL MEDICINE - PhD PROGRAM IN SCIENCES & MEDICAL BIOTECHNOLOGY – XXXth cycle
Coordinator: Prof. Emanuele Albano

Effect of unacylated ghrelin on dystrophic muscle in vivo

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SCIENTIFIC BACKGROUND

Muscle dystrophy is a group of diseases characterised by progressive muscle wasting. With an approximative frequency of 1 in 3500 live male births, the most common and lethal among them is Duchenne muscular dystrophy (DMD), an X-linked recessive disorder caused by mutations in the gene encoding cytoskeletal protein dystrophin (Moser, 1984; Koenig *et al*., 1988). Dystrophin connects F-actin in the subsarcolemmal cytoskeleton to the laminin in extracellular matrix (ECM) (Ervasti and Campbell, 1993) though stabilizing the fiber membrane during contractions. The mutation in dystrophin results in a frameshift error and production of non-functional protein unable to effectuate the connection between cytoskeleton and ECM. This leads to the damage of muscle fibers with subsequent necrosis. Partial regeneration of the damaged muscle occurs thanks to satellite cells - stem cell muscle progenitors, but the constant process of degeneration and regeneration leads to the exhaustion of satellite cell pool and produces a larger, but weaker muscle where the muscle is slowly replaced by fibrotic tissue.
Nowadays, the treatment of muscle dystrophy does not provide any resolutive method and focuses mainly on the suppression of the inflammation by use of glucocorticoids, though offering only limitated beneficial effects for the patients. Moreover, corticosteroid use is known to have adverse side-effects, such as behavioral changes, immune suppression, hypertension, development of central adiposity, insuline resistance, glucose intolerance, and bone demineralization (Schacke et al., 2002; Rockall et al., 2003; Merlini *et al*., 2012) limiting its lengh-of-use and effectiveness. Prednisolone, which is the standard-of-care for DMD boys, was shown to increase cardiac damage seen as collagen deposition and deterioration in function in mdx mice (Guerron *et al*., 2010; Janssen *et al*., 2014). Therefore is needed to search for novel treatments for DMD. Several new strategies have been already proposed (exon skipping, upregulation of utrophin, myogenic cell transplantation). Nevertheless, these therapies represent a major challenge because muscle is the most abundant tissue in the body.
Ghrelin is a small circulating peptide released mainly by cells in stomach in two forms – acylated (AG) and unacylated (UnAG). AG binds to growth hormone secretagogue receptor 1a (GHSR1a) and mediates growth hormone release, increased food intake, and adiposity (Kojima *et al*., 1999; Tschop *et al*., 2000; Theander-Carillo *et al*., 2006). In nephrectomized animals, AG increases cytochrome C oxidase and PGC-1α and PGC-1β activity though preventing muscle wasting through Akt phosphorylation (Barazzoni *et al*., 2010; Tamaki *et al*., 2015).
More abundant UnAG, together with AG, binds to a yet not identified receptor distinct from GHSR1a and promotes survival, differentiation and proliferation of myoblast, and has an anti-apoptotic effect on cardiomyocytes and endothelial cells *in vitro* (Baldanzi *et al*., 2002; Filigheddu *et al*., 2007; Togliatto *et al*., 2013). UnAG was shown to protect skeletal muscle cells *in vivo* from atrophy induced by fasting or denervation and to induce regeneration after hindlimb ischemia via p38/mitogen-activated signalling (Porporato *et al*., 2013; Togliatto *et al*., 2013). The research of last years demonstrated the antioxidant

role of UnAG in different cell types (Zhang *et al*., 2010; Zhang *et al*., 2011; Togliatto *et al*., 2013; Dieci *et al*., 2014). In transgenic mice, UnAG reduces inflammatory cytokines and reactive oxygen species (ROS) production (Cappellari *et al*., under revision). UnAG also promotes the satellite cell self-renewal, protecting their pool from exhaustion in pathological conditions of dystrophy.
Based on the observations *in vitro* and *in vivo* on muscle regeneration, reduced inflammation and together with our unpublished data demonstrating enhanced satellite cell self-renewal, UnAG seems to have therapeutic potential for muscle wasting that occurs in conditions of cancer cachexia, aging or muscle dystrophy. This project focuses on description of UnAG effect on muscle *in vivo* in mdx mouse, which is the most effective model of DMD, for characterizing the structural and functional properties of therapeutic interventions.

AIM OF THE PROJECT

- to asses effect of unacylated ghrelin on skeletal muscle *in vivo* in mdx mice

EXPERIMENTAL PROCEDURES

Mice

C57BL/6-*Myh6/Ghrl* transgenic (Tg) mice over-expressing the ghrelin gene in the heart and characterized by high levels of circulating UnAG and unchanged AG levels (Porporato *et al*., 2013) were crossed with dystrophin-deficient mdx mice (C57BL/ScSn-Dmdmx/J) obtaining mdx Tg- and mdx Tg+ animals. The genotype was determined by PCR.

Grasping test

Mice were gently lifted by the tail and allowed to grasp a grid connected to an ordinary electronic balance. While grasping, the animal continued to be lifted by the tail with increasing firmness until it lost its grip. At this precise moment, the values shown by the balance were recorded. The measurement was repeated 5 times.

Hanging wire test

Hanging wire test is a functional test for measuring muscle force and endurance. The animals underwent 3 min hanging starting with 10 points, where every fall was scored by -1 point and every reach by +1 point.

Latency to fall

Latency to fall is the longest time between two falls measured during hanging wire test.

RESULTS

The data were collected at several time points found in the literature as breaking points of the dystrophic phenotype – 1, 3 and 6 months of age. Functional tests were performed also at 4 and 5 months to capture the exact moment of changes in physical condition that follow the histological and biological occurrence.

1 month: Upregulation of unacylated ghrelin improves muscle regeneration

The cross-sectional area (CSA) measurement of quadriceps showed a shift to larger fibers in mdx Tg+ animals (Fig. 1). This can be explained or as improved regeneration or impaired degeneration of skeletal muscle due to high UnAG levels since at 3 weeks of age occurs the first degeneration/regeneration wave in mdx phenotype.

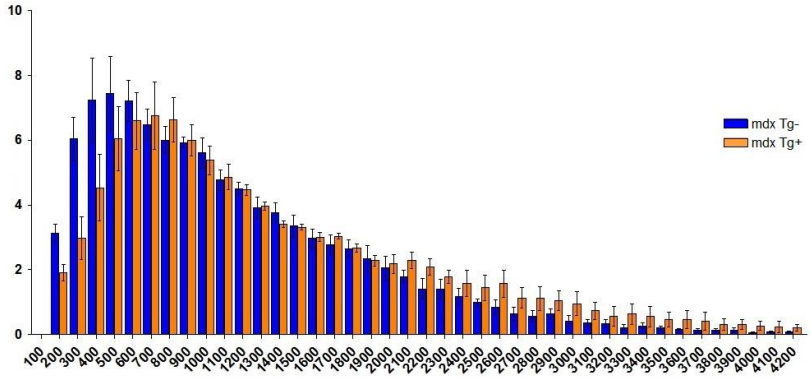

Fig. 1: Measurement of CSA of quadriceps of 1-month-old mdx Tg- and mdx Tg+ animals (N=3).

3 months: Unacylated ghrelin hampers skeletal muscle degeneration

Histological analysis at 3 months showed decreased necrosis of muscle fibers in diaphragm (Fig. 2A; $P<0.05$ vs. mdx Tg-) and quadriceps (Fig. 2B), seen as lower Evans Blue Dye (EBD) uptake. High levels of UnAG also protects diaphragm from collagen deposition (Fig. 2C) though slowing down the degeneration of skeletal muscle and its replacement by fibrotic tissue.

Muscle functionality was tested by grasping test, and mdx Tg+ animals showed statistically significant longer average holding time ($P<0.05$) compared to mdx Tg- mice (Fig. 2D).

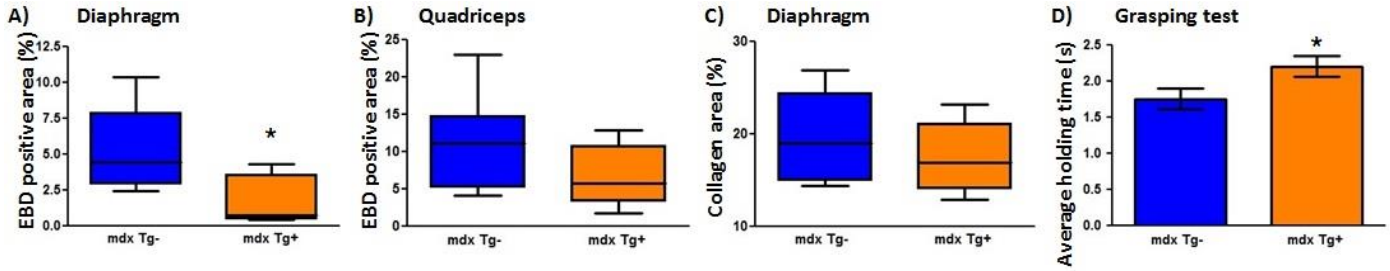

Fig. 2: Animals were injected intraperitonealy with 5 ul/1 g 1% EBD to visualize necrotic areas. EBD positive area of 3-months-old mdx Tg- and mdx Tg+ animals in **A)** diaphragm ($N_{mdx\ Tg-}=7$; $N_{mdx\ Tg+}=6$), *$P<0.05$ vs. mdx Tg-, and **B)** quadriceps ($N_{mdx\ Tg-}=7$; $N_{mdx\ Tg+}=6$). **C)** Measurement of collagen deposition by trichromic staining in diaphragm of mdx Tg- and mdx Tg+ animals (N=6). **D)** Average holding time in grasping test of 3-month-old mdx Tg- and mdx Tg+ animals ($N_{mdx\ Tg-}=22$, $N_{mdx\ Tg+}=18$), *$P<0.05$ vs. mdx Tg-.

4 months: Upregulation of UnAG in mdx dystrophic mice improves muscle function

At 4 months of age, we tested the muscle function of mdx Tg- and mdx Tg+ animals by hanging wire test. Obtained results show strong improvement in muscle function viewed as higher average score of mdx Tg+ animals compared to mdx Tg- (Fig. 3A) statistically significant from 80 s. Mdx Tg+ mice shown also statistical difference in the latency to fall – a time between two falls measured during hanging wire test (Fig 3B). This can be translated as bigger strength of mdx Tg+ muscles protected from degeneration by high levels of UnAG.

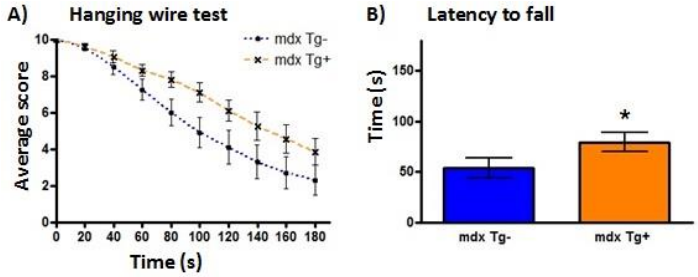

Fig. 3: A) Average score trend in hanging wire test of 4-months-old mdx Tg- and mdx Tg+ mice. Final score is represented (N=21). **B)** Average latency to fall of 4-months-old mdx Tg- and mdx Tg+ mice (N=21), *P<0.05 vs. mdx Tg-.

5 months: Upregulation of UnAG in mdx dystrophic mice improves muscle function

Similarly to 4 months, at 5 months we saw improvement of muscle function measured as higher average score (Fig. 4) and longer time between two falls (data not shown) during hanging wire test of mdx Tg+ animals compared to mdx Tg-.

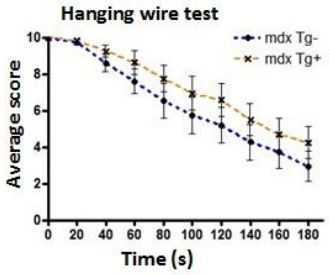

Fig. 4: Average score obtained in hanging wire test of 5-monts-old mdx Tg- and mdx Tg+ animals, final score is represented ($N_{mdx\ Tg-}$=18; $N_{mdx\ Tg+}$=19).

6 months: Upregulation of UnAG leads to decrease in collagen deposition and improved muscle function

At 6 months of age, due to continuous cycles of degeneration and impaired regeneration, mdx muscles are slowly replaced by fibrotic tissue. Although the collagen area in diaphragm enlarged from 3 to 6 months of age (Fig. 2C, 5A), upregulation of UnAG significantly lowered collagen deposition in mdx Tg+ compaired to mdx Tg- animals. Histological analysis revealed lower EBD uptake in quadriceps of mdx Tg+ animals indicating decreased necrosis (Fig. 5B) of muscle fibers.

Similarly to previous time points, 6-months-old mdx Tg+ performed better in hanging wire test (Fig. 5C) reaching higher average score and longer time in latency to fall (Fig. 5D).

Interestingly, the weight normalized to tibial lenght was increased in gastrocnemius (Fig. 5E), quadriceps (Fig. 5F) and extesor digitorum longus (data not shown) of mdx Tg- compaired to mdx Tg+ animals probably due to compensative hypertrophy present in dystrophic muscles as a result of damage. Moreover, the weight of mdx Tg+ muscles was similar to the one of WT (data no shown). But the

analysis of Feret diameter distribution of these muscles did not show any difference between mdx Tg- and mdx Tg+ mice (Fig. 5G-H). Therefore, the difference in the weight of these muscles in mdx Tg- could be explained by increased fibrosis or fat deposition.

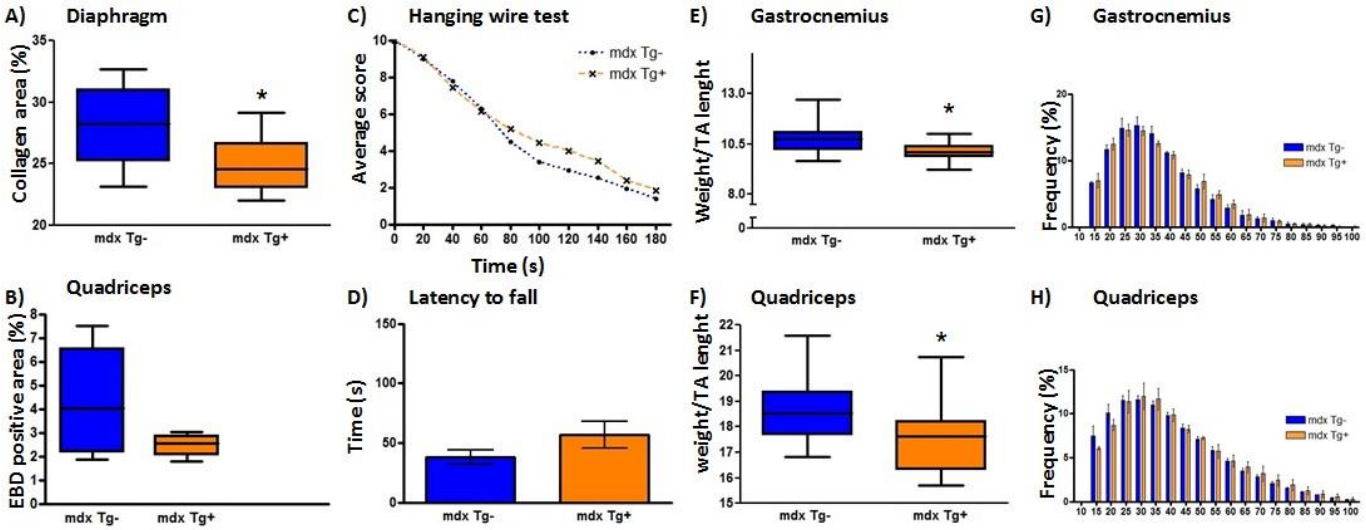

Fig. 5: A) Collagen deposition measured by trichromic staining in diaphragm (N=7), *P<0.05 vs. mdx Tg-. **B)** EBD positive area in quadriceps of 6-months-old mdx Tg- and mdx Tg+ animals (N=4). Animals were injected intraperitonealy with 5 ul/1 g 1% EBD to visualize necrotic areas. **C)** Hanging wire test of 6-months-old mdx Tg- and mdx Tg+ mice ($N_{mdx\ Tg-}$=23; $N_{mdx\ Tg+}$=20). **D)** Average latency to fall of 6-months-old mdx Tg- and mdx Tg+ mice ($N_{mdx\ Tg-}$=23; $N_{mdx\ Tg+}$=20). Muscle weight of **E)** gastrocnemius (N=12) and **F)** quadriceps ($N_{mdx\ Tg-}$=13; $N_{mdx\ Tg+}$=12) of 6-months-old mdx Tg- and mdx Tg+ animals normalized to tibial lenght, *P<0.05 vs. mdx Tg-. Feret diameter distribution of **G)** gastrocnemius (N=3) and **H)** quadriceps (N=3) of 6-months-old mdx Tg- and mdx Tg+ animals.

CONCLUSION

Taken together, these data show positive effect of high levels of circulating UnAG on muscle condition in mdx murine model, observed as lower EBD uptake and lower collagen deposition in studied muscles, and overall better functionality in grasping test, hanging wire test and latency to fall. This suggests that UnAG could be seen as a novel approach to treat muscular dystrophies or alone or as an adjuvant therapy. UnAG also triggers decrease in ROS and cytokine production (Cappellari *et al*., under revision) therefore reducing the inflammation and creating more enhancing environment for regeneration process enhancing satellite cell self-renewal and protecting their pool from the depletion.

Cardiac dysfunction is often given only a secondary focus, however, it´s a critical component of DMD progression because the lethality of DMD for human consist in respiratory or cardiac failure (Moser, 1984; Emery and Muntoni, 2003). As previously described, mdx mice develop cardiac fibrosis and increased expression of procollagen type I and III in heart leading to diastolic and systolic dysfunction and also acute heart failure is evident (Yasuda *et al*., 2005; Au *et al*., 2011). Since it was previously shown that UnAG inhibit apoptosis in cardiomyocytes *in vitro* (Nagaya *et al*., 2001; Baldanzi *et al*., 2002; Okumura *et al*., 2002; Nagaya *et al*., 2004; Rizzo *et al*., 2013), it is necessary also to study the effect of UnAG also on cardiac muscle *in vivo* and compare obtained results with treatment by glucocorticoids, that are now considered as standard-of-care for DMD but were shown to have detrimental effect on cardiac tissue, to show a possible advantage in use of UnAG alone or in combination with standard drugs.

ACKNOWLEDGMENTS

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- 22/09/2015 The Biomania Student Scientific Meeting 2015 (Brno Observatory and Planetarium, Brno, Czech Republic)

POSTERS

Simone Reano, Elia Angelino, Michele Ferrara, **Hana Sustova**, Emanuela Agosti, Andrea Graziani, Nicoletta Filigheddu. Exploring the therapeutics potential of unacylated ghrelin in muscle dystrophy. MDA scientific conference, Washington DC, USA. March 11-14, 2015.

Emanuela Agosti, Elia Angelino, Simone Reano, **Hana Sustova**, Michele Ferrara, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu.Protective effects of unacylated ghrelin in aging mice. Brallo-Pavia, Italy. June 8-12, 2015.

Hana Sustova, Simone Reano, Elia Angelino, Emanuela Agosti, Michele Ferrara, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Positive effects of unacylated ghrelin on dystrophic muscle in vivo. Biochemical PhD students meeting. Brallo-Pavia, Italy. June 8-12, 2015.

Elia Angelino, Simone Reano, Michele Ferrara, **Hana Sustova**, Emanuela Agosti, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Unacylated ghrelin enhances satellite cells activity and promotes skeletal muscle regeneration. International spring research day. Lugano-Vezia, Switzerland. June 19, 2015.

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