ANNUAL REPORT - DEPARTMENT OF TRANSLATIONAL MEDICINE - PhD PROGRAM IN SCIENCES & MEDICAL BIOTECHNOLOGY - XXX cycle (2015-2018)
Coordinator: Prof. Emanuele Albano

Protective Effects of Unacylated Ghrelin in Aging Mice

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SCIENTIFIC BACKGROUND

Aging often correlates with a progressive loss of physiological functions that, in human, causes an increased susceptibility to a number of diseases (Lòpez-Otin et al., 2013). Cross-sectional studies show a reduction of muscle mass and strength from the third or fourth decade onwards (Janssen et l., 2000; Silva et al., 2010), indicating that muscle tissue is involved in this decline. This degeneration, which leads to an irreversible loss of skeletal muscle mass and functionality in aged individuals, is defined as sarcopenia.

Sarcopenia is a multifactorial syndrome that results in frailty, mobility disorders, loss of independence, and high risk of mortality (Cruz-Jentoft et al., 2010; Cederholm at al., 2013). The underlying mechanisms and etiology of sarcopenia remain poorly defined, but include hormonal changes, decrease physical activity, chronic inflammation, oxidative damage, insulin resistance and nutritional deficiency. The age-related decrease of anabolic factor, such as GH and IGF-1, testosterone and estrogen, and parallel increase of catabolic factors, such as pro-inflammatory cytokines (Barzilai et al., 2012; Shaw et al., 2013), generates an imbalance between the rates of muscle protein synthesis and muscle proteolysis, in which net muscle protein balance is negative (Breen et al., 2011; Yarasheski, 2003). This condition leads to the development of muscle atrophy.

In addition to atrophy, the deterioration of skeletal muscle involves age-impaired muscle regeneration. Muscle regeneration is sustained by myogenic precursors called satellite cells, located underneath the basal lamina of the myofibers and responsible for muscle homeostasis (Collins et al., 2005).
Normally, satellite cells are quiescent and characterized by the expression of the transcription factor paired-box 7 (Pax7) (Seale et al., 2000). After injury, different signals from the environment of damaged muscle trigger the activation and proliferation of satellite cells that start to express myogenic genes, such as the myogenic factor 5 (Myf5) and the myoblasts determination factor (MyoD) (Singh and Dilworth, 2013). After a proliferative phase, myoblasts undergo differentiation, accompanied by reduction in the expression of Pax7 and increase of myogenin and MRF4 (Dumont et al., 2015). At this stage, myoblast fuse into existing damaged myofibers or together to create *de novo* myofibers (Yin et al., 2013). Even after multiple injuries, the pool of satellite cells is maintained constant thanks to a mechanism called self-renewal (Collins et al., 2005). Indeed, a minor fraction of activated satellite cells gives rise to quiescent $Pax7^{+}/MyoD^{-}$ cells as a consequence of asymmetric division (Kuang et al., 2007).

The lower regenerative potential during aging correlates with the reduction of the pool size of satellite cells and with the decline of their function (Jang et al., 2011; Verdijk et al., 2014). Aged mammals show a reduction of proliferation and differentiation of satellite cells (Lees et al., 2006; Corbu et al., 2010) and, above all, their abilities to exert self-renewal through asymmetric division (Bernet et al., 2014). Moreover functional changes in satellite cells can be consequence to age-related modification of their niche (Parker 2015). On the long run, aged muscles replace damage tissue with fat deposition and collagen (Budui et al., 2015).

Acylated and unacylated ghrelin (AG and UnAG, respectively) are circulating peptide hormones generated by the ghrelin gene mainly in the stomach in consequence of fasting or caloric restriction (Kojima et al., 1999; Asakawa et al., 2001). AG, through binding to growth hormone secretagogue receptor type 1a (GHSR-1a), induces strong release of GH, stimulates food intake, adiposity and positive energy balance (Kojima et al., 1999; Tschöp et al., 2000). Acylation of ghrelin is essential for its binding to GHSR-1a, since the unacylated form does not activate this receptor and is devoid of any GH-releasing activity (Kojima et al., 1999) and therefore UnAG has been considered for many years the inactive product of AG catabolism (Chen et al., 2009).
However, both peptides share common activities, mediated by an unknown receptor, on skeletal muscle where they protect against atrophy, caused by burning, dexamethasone, denervation and fasting, and promote proliferation and fusion of myoblast (Sheriff et al., 2011; Porporato et al., 2013; Filigheddu et al., 2007). In addiction UnAG promotes regeneration of skeletal muscle following hindlimb ischemia (Togliatto et al., 2013). Moreover, ghrelin can ameliorate the cachectic state in animal models of cancer cachexia and in cisplatin-induced and angiotensin-II-induced muscle wasting (Akamizu et al., 2012; Garcia et al., 2013; Sugiyama et al., 2012; Chen et al., 2015). The molecular mechanism by which AG/UnAG prevent muscle wasting involved PI3Kb/mTORC2/Akt, p38 and, only for AG, activation of GH/IGF-1 axis mediated by GHSR-1a (Reano et al., 2014).

Aging is characterized by modification of AG/UnAG pathway, due to decrease in plasmatic levels of these hormones in human or decline in receptor or post-receptor functions in animal models (Yin and Zhang, 2015). As a result of this downregulation, we hypothesize that AG/UnAG may play a protective role against sarcopenia since AG/UnAG protect against atrophy (Sheriff et al., 2011; Porporato et al., 2013) and ROS-induced cell damage and promotes muscle regeneration (Togliatto et al., 2013).

AIMS OF THE PROJECT

Aim of the project is to assess if UnAG could have protective effects against the development of sarcopenia. Our proposal is mainly focused on UnAG because, unlike AG, its activity does not involved GHSR-1a activation. Decrease of GH/IGF-1 axis is indeed associated with extended longevity (Garinis et al., 2008). Moreover, UnAG permit to avoid potential adverse metabolic side effect of AG, including the induction of insulin resistance, diabetes, and stimulation of fat accumulation (Dezaki 2013; Müller et al., 2015).

EXPERIMENTAL PROCEDURES

Mice

All experiments were conducted on old FVB mice (12 and 24 months). In Myh6/Ghrl transgenic mice, the overexpression of Ghrl gene under the control of the cardiac-specific promoter of alpha-myosin heavy chain (Myh6) gene results in a 50-fold increase of circulating UnAG, without affecting AG levels (Porporato et al., 2013).

Analysis of the plasmatic levels of AG and UnAG

Plasmatic levels of AG and UnAG were analyzed at different ages (3 months, 1 year, 2 years) with the Acylated or Unacylated Ghrelin Express EIA kit (Spibio, Bertin Pharma) following the producer's instructions.

Non-invasive functional test

Grasping test evaluates mouse limb strength, exploiting the natural tendency of the mice to grip to a bar when suspended by the tail. For this test, the mouse grasps on a horizontal bar while is pulled by the tail. The bar is attached to a force transducer that peak pull-force achieved on a digital display. Each animal was tested 5 times, and the average value of the maximum weight that the animal managed to hold was recorded and normalized to the mouse's weight.

Hanging wire test measures global 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 one of the side of the wire by +1 point. Data are expressed as average score for every time point. Moreover, during the same test it is possible to analyze "latency to fall", namely the longest time between two falls.

Tissue collection

At the age of 2 years, epididymal fat, heart, liver, spleen, gastrocnemius, tibialis anterior, quadriceps, soleus and extensor digitorum longus muscles were collected from WT and Tg mice, weighted and snap-frozen.

Gene expression

Total RNA was extracted from gastrocnemius of WT and Tg mice using RNAzol (Sigma Aldrich) and retrotranscribed with High-Capacity cDNA Reverse Transcription Kit, (Ambion, Life Technologies). Gene expression was evaluated by real time PCR using Taqman probes for Myostatin (Mm01254559_m1, Life Technologies), MuRF-1a (Mm0115221_m1, Life Technologies) and beta-actin (Mm01205647_ g1, Life Technologies).

Cross sectional area

Serial transverse cryosections (7 µm thick) of the midbelly region of tibialis anterior of WT and Tg mice were cut at –20°C and mounted on glass slides. The sections were air-dried, fixed for 10 minutes in 4% paraformaldehyde and stained with anti-laminin antibody (Dako). Images of whole muscle sections were acquired with the slide scanner Pannoramic Midi 1.14 (3D Histech) and cross-sectional areas (CSA) of fibers quantifies with ImageJ software (v1.49o).

RESULTS

Plasmatic levels of AG/UnAG increase during aging.

Since ghrelin signaling is modified in aging (Yin and Zhang, 2015), we analyzed the levels of AG and UnAG in WT mice of different ages: 3 month, 1 year and 2 years and we observed that the plasmatic levels of both AG and UnAG increase with aging (Figure 1). We hypothesize that this increase may represent a compensatory mechanism to the decline in receptor or post-receptor functions (Yin and Zhang, 2015).

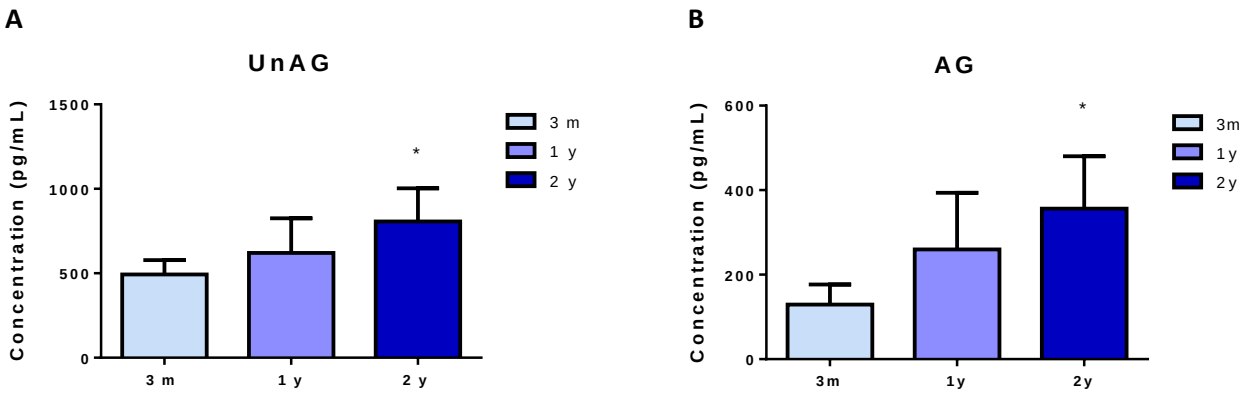
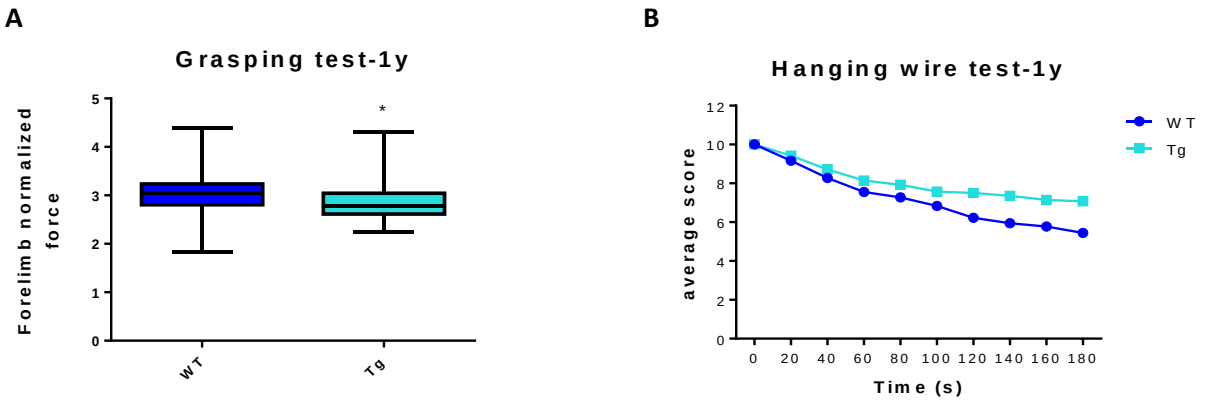


Figure 1. AG and UnAG increase during aging.

Plasmatic levels of UnAG (A) and AG (B) in WT FVB mice of 3 months, 1 year and 2 years, determined by Acylated or Unacylated Ghrelin Express EIA kit (Spibio, Bertin Pharma) (3m N = 5, 1y N = 8; 2y N = 6).

Constitutively high levels of UnAG improve muscle functionality.

Aging correlates with reduced muscle functionality that results in mobility disorders and loss of independence. To investigate the putative effects of UnAG, described as anti-atrophic hormone (Sheriff et al., 2011; Porporato et al., 2013), on this phenomenon, we used two different kinds of test: grasping test, which allows to evaluate grip strength, and hanging wire test, which analyzes global muscle force and endurance. 1-year-old Tg mice showed a slightly reduced grip force compared to WT (Figure 2A) but, despite this, they are characterized by better physical condition. Indeed, Tg mice performed better in hanging wire test (Figure 2B) and they showed a significantly increased latency time between two successive falls (Figure 2C). Preliminary results on 2-years-old mice demonstrated that constitutively high levels of circulating UnAG protect aged muscle from loss of strength. Indeed, Tg mice are characterized by significantly higher grip force compared to WT mice of the same age (Figure 2D).



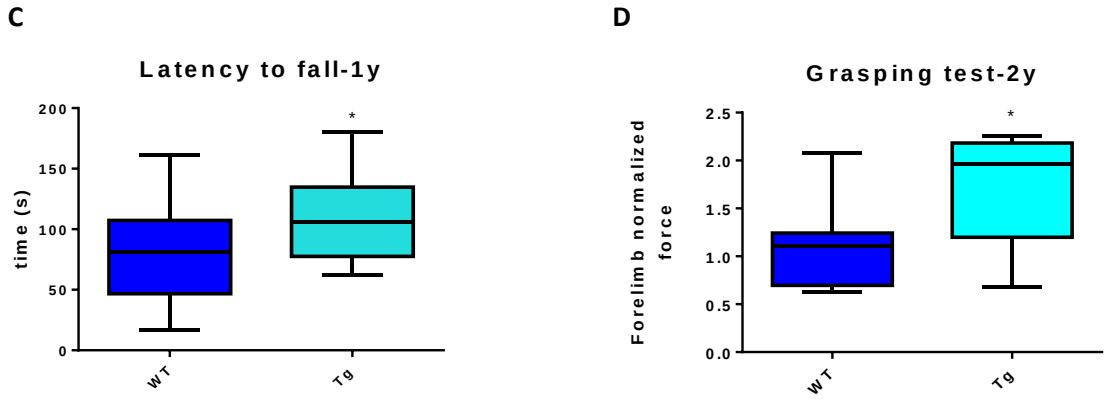
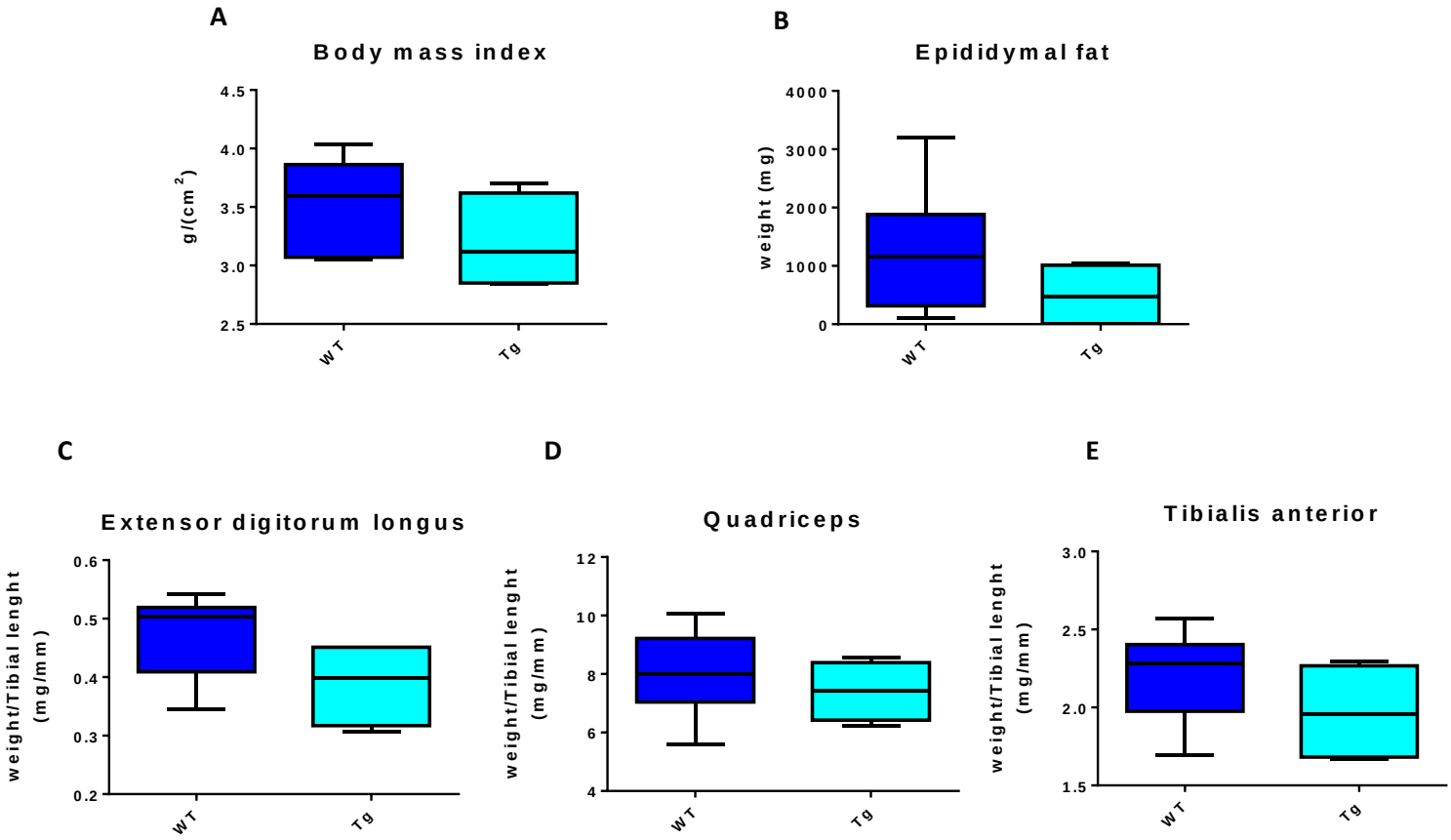


Figure 2. Tg mice show better muscle functionality compared to WT.
Forelimb normalized force in grasping test in 12 (A) and 24-months-old (D) WT and Myh6/Ghrl transgenic mice. * p < 0.05 vs WT (12 months WT N = 19; Tg N = 18; 24 months WT N = 6; Tg N = 4). Average score trend in hanging wire test of 12-months-old WT and Myh6/Ghrl transgenic mice (WT N=13; Tg N=11) (B). Average latency to fall of 12-months-old WT and Myh6/Ghrl transgenic mice – the longest time between two falls measured during hanging wire test. *p < 0.05 (WT N=13; Tg N=11) (C).

Myh6/Ghrl transgenic mice show a slight decrease in body mass index, muscles weight and body fat accumulation.

Aging is characterized by body fat accumulation and impaired muscle regenerative abilities that can lead to muscle mass loss and to the substitution of myofibers with ectopic tissues including fat (Sciorati et al., 2015). 2-years-old Tg mice showed a slight decrease in body mass index compared to WT (Figure 3A), reflecting the trend toward lower body fat deposition (Figure 3B). Moreover, even if tissues collected from Tg mice do not show significant differences compared to WT mice, the muscles of Myh6/Ghrl mice show a mild decrease in weight (Figure 3C-G, data not shown).



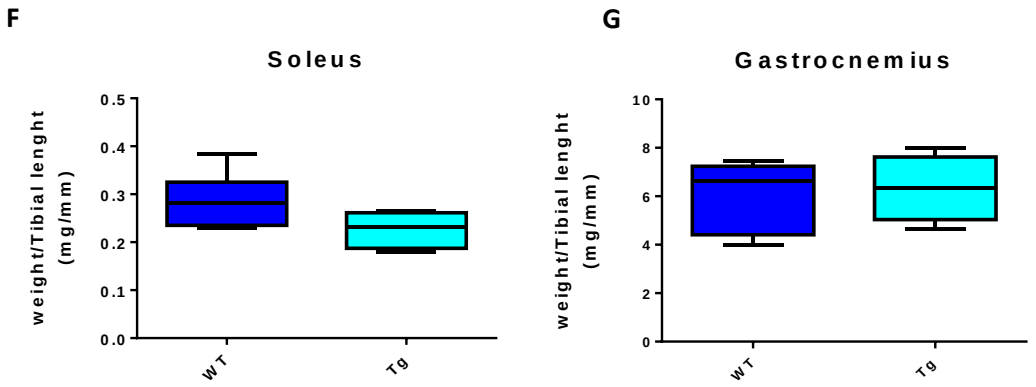


Figure 3. Myh6/Ghrl transgenic mice show a slightly decrease in BMI, body fat accumulation and muscle weight.
Body mass index (A) and weight of epididymal fat pad (B) were measured in 24-months-old WT and Myh6/Ghrl transgenic mice. Weight of extensor digitorum longus (C), quadriceps (D), tibialis anterior (E), soleus (F) and gastrocnemius (G) were measured in 24-months-old WT and Myh6/Ghrl transgenic mice. WT N=6; Tg N=4.

Myh6/Ghrl transgenic mice are protected against atrophy.

Since atrophy is one of the principal causes of skeletal muscle mass loss in aged individuals, we wanted to verify if UnAG, which shows anti-atrophic activity in different conditions, maintained this activity also in aging (Sheriff et al., 2011; Porporato et al., 2013). Cross-sectional area (CSA) distribution of tibialis anterior from WT and Tg mice, shows a mild shift towards bigger areas in Tg muscles, indicating that they are slightly protected against muscle mass loss. Then, we examined the expression of MuRF-1 and Myostatin (MSTN), an atrogene upregulated in atrophy (Bodine et al., 2001) and a negative regulator of muscle growth (McPherron et al., 1997) respectively, obtaining a mild decrease in Tg mice compared to WT counterpart. Taken together, these results prove that the slight decrease in muscle weight is not due to muscle atrophy since Tg mice have better CSA distribution than WT and they additionally show a down-regulation of the expression of MuRF-1 and Myostatin.

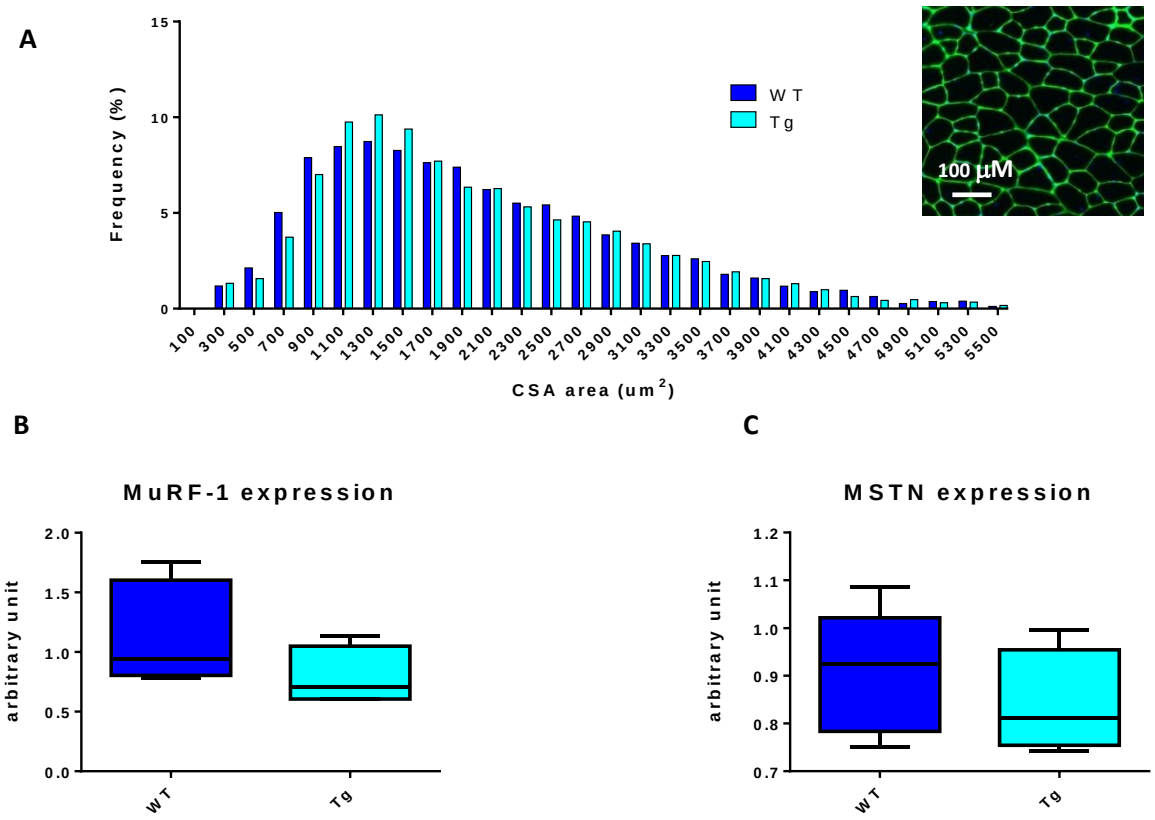


Figure 4. UnAG protects against atrophy.
Frequency distribution of CSA of myofibers in tibialis anterior muscles from WT and Myh6/Ghrl transgenic mice; WT N=6; Tg N=4. (A) In the inset, representative image of cross section of tibialis anterior fiber stained with Ab against a-laminin. MuRF1 (B) and Myostatin gene expression (C) was determined by real-time RT-PCR in gastrocnemius of 24-months-old WT and Myh6/Ghrl transgenic mice; WT N=6; Tg N=4.

CONCLUSIONS

Ghrelin receptor functions and/or post-receptor pathways declines in old mice (Akimoto et al., 2012). We have demonstrated that plasmatic levels of AG and UnAG are significantly increased in aged FVB mice (Figure 1), so it is possible to speculate that this augmented production represents a compensatory response to the downregulation of ghrelin signaling.
Upregulation of UnAG production, overcoming its signaling pathway decline, could represent a protective mechanism against age-induced atrophy and impaired muscle regeneration. We achieved upregulation of circulating UnAG by myocardial *Ghrl* overexpression in Myh6/Ghrl mice (Porporato et al., 2013).

Preliminary results demonstrate that overexpression of UnAG improves muscle functionality in old mice. Even if 1-years-old Tg mice are characterized by lower grip force (Figure 2A), they show an improvement of general muscle force and endurance compared to WT mice (Figure 2B-C). Moreover, UnAG overexpression results in greater strength of grip compared to WT mice of the same age (Figure B).
Tg mice showed a trend in atrophy protection and lower body fat accumulation that will be confirmed in the future, increasing the population of mice included in the study, as the preliminary results here reported were obtained on a very small number of mice.

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LESSONS

- "Tissue engineering: the state of the art" – 14th November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont.

- "Regenerative Medicine" – 21st November 2014 – Prof. Maria Prat - Department of Health Sciences, University of Eastern Piedmont.

- "Ribosomopathies" – 25th May 2015 – Prof. Steve Ellis – Medical School, University of

SEMINARS

1. “Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders” – 06th November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda

2. “Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention” – 17th November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.

3. “La scoperta del bosone di Higgs” – 25th November 2014 - Dott. Roberta Arcidiacono - DiSCAFF, University of Eastern Piedmont - Dott. Marta Ruspa - Department of Health Sciences, University of Eastern Piedmont.

4. “Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori” – 27th November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women’s Health, Paris.

5. “Humoral responses to HCV infection and clinical outcomes” – 28th November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow

6. “Uncovering the role β-HPV in field cancerization: a collaboration in progress” – 4th December 2014 – Prof. Girish Patel, Cardiff.

7. “Focus on deliver: from basics of NAFLD to hot topics in HBV & HCV infections” – 5th December 2015 – Prof. Rifaat Safadi M.D.

8. “From the legend of Prometheus to regenerative medicine” – 11th December 2015 – Prof. Antonio Musarò

9. “Pregi e difetti dei nuovi anticoagulanti orali nella pratica clinica” – 16th December 2015 – Prof. Giancarlo Agnelli

10. “Microglia microvescicles: messengers from the diseased brain” – 17th December 2014 - Dott. Roberto Furlan, San Raffaele University, Milan.

11. “Anticancer strategy Targeting cancer cell metabolism in ovarian cancer” – 19th January 2015 - Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University.

12. “Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states” – 20th January 2015 - Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive “L. Spallanzani” IRCCS, Rome.

13. “Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis” – 21st January 2015 - Prof. Valeria Poli - Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin.

14. “Myeloid cells as therapeutic target in cancer” – 27th January 2015 - Prof. Antonio Sica -

15. “Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells” – 11th March

2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.

16. “Signal control in iNKT cell development and function” – 09th April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).

17. “Actin-based mechanisms in the control of gene expression and cell fate” – 21st April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).

18. “An integrated approach to the diagnosis and treatment of ovarian cancer” – 7th May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).

19. “Conflicting interests and scientific communication” – 14th May 2015 – Prof. Kathleen Ruff – RightOnCanada Founder, Senior Advisor to the Rideau Institute (Ottawa, Canada).

20. “Recent developments in (cutaneous) Human Polyomavirus research” – 5th June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).

21. "NF-κB signaling in myogenesis and muscle diseases" – 26th June 2015 – Prof. Denis C. Guttridge – Human Cancer Genetics Program, Department of Molecular Virology Immunology and Medical Genetics, The Ohio State University (Ohio, USA)

22. Miniworkshop on “Biotechnology for Dermatology” – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France.

23. “High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach” – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).

24. “Le cellule staminali nel danno renale acuto e nel trapianto di rene” – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).

25. “Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)” , “Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)” – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).

CONGRESS

“27° riunione nazionale “A. Castellani” dei Dottorandi di ricerca in Disclipline Biochimiche”, Brallo di Pregola (IT), Università degli studi di Pavia, 8-12 June 2015.

PUBLICATIONS

Angelino E, Reano S, Ferrara M, **Agosti E**, Graziani A, Filigheddu N. (2015). Antifibrotic activity of acylated and unacylated ghrelin. Int J Endocrinol. 2015:385682.

POSTER

Simone Reano, Elia Angelino, Michele Ferrara, Hana Sustova, **Emanuela Agosti**, Sara Clerici, Nicoletta Filigheddu. Unacylated ghrelin stimulates satellite cells self renewal and skeletal muscle regeneration. EMBO workshop molecular mechanisms of muscle growth and wasting in health and disease. Ascona, Switzerland. September 20-25th, 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. Brno, Czech Republic. The biomania student scientific meeting 2015. September 22th, 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 19th, 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 8th-12th, 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 8th-12th, 2015.

Simone Reano, Elian 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 11-14th, 2015.