Final report for PD OTKA 128370 (2019-2021)

Carotenoids - precursors of vitamin A - are natural pigments found in many fruits and legumes and are known to have a very similar chemical structure to retinol [1]. Unlike beta-carotene, astaxanthin is able to readily cross the blood-brain barrier, however it cannot be converted to retinol, thus to vitamin A and therefore cannot support retinol-specific processes such as vision [2].

Astaxanthin (AX) is extensively produced by krill, arctic shrimp, and algal species such as *Haematococcus pluvialis*, and also by the yeast *Phaffia rhodozyma* [3]. Our research group together with a muscle research team from the University of Szeged, led by prof. Anikó Keller Pintér published a review that summarizes the beneficial effects of this special fat-soluble carotenoid with strong antioxidant capacity, including its impact on muscle physiology (**Sztretye et al. 2019**). There are cumulating data suggesting that AX can reduce oxidative stress and prevent against mitochondrial damage by permeating through mitochondrial membranes, which is an unique feature among antioxidants [4,5]. AstaReal A1010, an astaxanthin-rich natural micro algal product, consisting of crushed and spray-dried aplano-spores of the green microalga *Haematococcus pluvialis* was a kind donation for research purposes from AstaReal Co., Ltd., (Nacka, Sweden).

In our first set of experiments we recruited eighteen C57Bl6 male 4-6 months old mice that were separated randomly into two groups. AX diet (per os) lasted for 4 weeks (AX group) while littermates were fed with standard rodent chow (CTRL group). The special chow was prepared with the addition of 4 g/kg of AstaReal A1010 (dissolved in 100% ethanol) to the standard rodent pellet for a final concentration of 0.02% AX. This concentration was chosen according to the literature [6].

Since AX is suggested to be used as a dietary supplement for healthy individuals, we aimed to describe its effects under physiological conditions on healthy young adult muscles. We demonstrated that AX improves grip force *in vivo* and tetanic force *in vitro* which occurs in the absence of changes in the excitation contraction (ECC) machinery. We provided evidence that mitochondrial calcium uptake during repetitive tetanic stimulation was reduced in the AX supplemented group as compared to the CTRL group. We also examined the effect of retinol - a structurally similar compound to AX - on the ECC mechanism and fatigability of fast twitch skeletal muscles and propose a model of AX actions. (**Sztretye et al. 2020a**). Apart from the metabolic aspects, which were only indirectly addressed in the paper, our results help to

understand the physiological consequences of antioxidant administration on healthy people. Overall, the data support the beneficial effects of AX in mammals and we believe that our results open up new treatment directions for patients suffering in metabolic diseases. We concluded that further studies are needed to understand the possible underlying pathways of AX actions both at cellular and at organ levels.

Small ankyrins (sAnk1) are muscle-specific isoforms encoded by the Ank1 gene that are involved in the organization of the SR of striated muscles. The volume of SR longitudinal tubules is significantly reduced in skeletal muscle fibers of sAnk1 knock-out (KO) mice. In collaboration with an Italian working group led by prof. Vincenzo Sorrentino, we investigated the consequences of the lack of sAnk1 protein in the skeletal muscles of mice. We found that the amplitude of Ca^{2+} transients induced by depolarizing pulses was decreased on sAnk1 KO muscle fibers. In parallel, the amplitude and spatial distribution of elemental Ca^{2+} release events (ECRE) were also significantly lower than in wild-type mice. These data suggest that the absence of sAnk1 results in a decrease in SR releasable calcium presumably through a decrease in Ca^{2+} storage due to a decrease in SR volume **(Pierantozzi et al. 2019**).

Store-operated Ca^{2+} entry (SOCE) is a physiological process that plays an important role in many cell types, and involves the entry of Ca^{2+} ions into cells that is activated by depletion of intracellular calcium stores. However, its role in physiological muscle activation, more specifically in skeletal muscles is rather controversial. To answer this question and elucidate this, we investigated the release of calcium from the sarcoplasmic reticulum (SR) in a mouse strain that has increased muscle mass but decreased muscle strength due to a mutation in the myostatin gene (Cmpt). We observed a significant decrease in the amount of calcium released in Cmpt mice, indicating a lower SR calcium content. The SR release also decreased more rapidly during muscle stimulation than in control animals, suggesting that Ca^{2+} replenishment was also hindered. To describe the phenomenon, we developed a simple model that assumed reduced SOCE as an important component of calcium influx across the surface membrane. With this, the decrease in the steady state of SR calcium content and the time course of SR release were well modeled [7]. To further confirm the role of SOCE, we performed experiments were the expression of the channel responsible for entry on the surface membrane (Orai1) was reduced after electroporation into the flexor digitorum brevis muscles of adult mice of a properly designed shRNA against Orai1. In the muscles treated in this way, SOCE was significantly reduced. In addition, our results also suggested that Orai1 channels are organized

into functionally distinct groups. Based on these findings, we proposed that SOCE plays a fundamental role in the defense against fatigue of skeletal muscles (**Sztretye et al. 2020b**).

We were also invited to write a detailed review in which mouse models available for muscle disease were presented (**Sztretye et al. 2020c**).

During aging or prolonged inactivity (for example: weightlessness, prolonged bed rest), a decrease in muscle strength - both muscle mass (quantity) and strength generation (quality) - is observed. Among other things, this is due to the associated mitochondrial dysfunction and increased oxidative stress. Increased oxidative stress leads to altered electromechanical contact and calcium homeostasis in elderly muscle. In addition, increased muscle loss, atrophy of residual muscle fibers, and dysfunction of satellite cells can also be observed. Oxidative stress is characterized by an alteration of the balance between pro-, and anti- oxidant compounds, leading to damage of the macromolecules and changes in redox signaling and cell function. Naturally occurring antioxidants prevent or slow down these processes, helping to prolong life. In our work, we investigated the effect of astaxanthin, one of the most common antioxidant xanthophyll carotenoids in marine organisms, on skeletal muscle function in elderly animals. Krill has emerged as a novel sustainable source of omega-3 polyunsaturated fatty acids (n-3 PUFAs); its oil form is remarkably rich in the long-chain PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are essential fatty acids for basic brain function. Moreover, krill oil contains bioactive ingredients such as AX, choline and essential amino acids which cannot be synthesized endogenously; nonetheless, krill oil has been described as extremely beneficial to brain fitness, cognition and memory [8]. In our next set of experiments, we have investigated the molecular mechanism by which AX and krill oil supplementation (4 weeks) influences skeletal muscle performance and exerts favorable effects on mitochondrial and cognitive function in aging mice. We propose that the administration of geroprotector nutraceuticals such as AX and krill oil would ameliorate the progression of aging by suppressing aging-induced muscle fitness loss and the subsequent cognition impairments. Due to its high-energy metabolism, both the human brain and the skeletal muscles are especially vulnerable to oxidative stress. In our paper, we hypothesized that krill oil being able to readily cross the blood–brain barrier and with its unique content of PUFAs could potentially counteract the negative effects of excessive ROS production and improve cognition (**Singlár et al. 2021**). Our results support the positive health outcomes of nutraceutical administration as we observed improved cognition and skeletal muscle function without major alterations of the excitation-contraction coupling and mitochondrial dynamics.

In our last set of experiments we aimed to analyze the actions of AX on appetite and body weight changes, as well as on the expression of the regulatory proteins affecting glucose homeostasis in skeletal muscles **(Gönczi et al. 2021**, submitted). Furthermore, we sought evidence whether cardiac excitability is affected by astaxanthin supplementation. We also hypothesized that AX affects the hypothalamic arcuate nucleus as an important center for food intake and metabolism. We found that chronic AX dietary supplementation increased food intake and led to increased body weight. This increased food intake is at least partially due to the enhanced inhibition of appetite-stimulating POMC neurons. Our data support the hypothesis that glucose metabolism is affected by AX in a way which exerts anti-diabetic actions but partially challenges the view on findings with altered food intake.

The results obtained during the the financing period were presented on a poster at the Biophysical Society 63rd Annual Meeting in Baltimore, California, USA (2019.03.02-2019.03.05), and also at the Gordon Research Conference Muscle: Excitation-Contraction Coupling in Lucca, Italy (2019.05.19-2019.05.24). Also, I was able to attend the Biophysical Society 64th Annual Meeting in San Diego, California, USA (2020.02.15- 2020.02.19) where I presented a poster (**Mónika T Sztretye**, Zoltán Singlár, László Szabó, Péter Szentesi, Beatrix Dienes, Mónika Gönczi, László Csernoch: Astaxanthin Improves Tetanic Force Without Altering Skeletal Muscle Excitation-Contraction Coupling in Mice. VOLUME 118, ISSUE 3, SUPPLEMENT 1, 408A, FEBRUARY 07, 2020). To conclude, the results obtained during this project were summarized and presented as an oral lecture at the online Conference on Therapeutical Purposes Research and Development II between September 23-24 2021 (https://pharm.unideb.hu/hu/conference-therapeutical-purposes-research-and-development-ii).

Papers published during the period of the PD OTKA 128370 project (2019-2021):

A. **M. Sztretye**, B. Dienes, M. Gönczi, T.Czirják, L. Csernoch, .L Dux, P. Szentesi and A. Keller-Pintér (2019) Astaxanthin: A Potential Mitochondrial-Targeted Antioxidant Treatment in Diseases and with Aging. *Oxid Med Cell Longev* 2019**.** https://doi.org/10.1155/2019/3849692.
B. Pierantozzi E, Szentesi P, Al-Gaadi D, Oláh T, Dienes B, **Sztretye M**, Rossi D, Sorrentino V, Csernoch L. (2019) Calcium Homeostasis Is Modified in Skeletal Muscle Fibers of Small Ankyrin1 Knockout Mice. *Int J Mol Sci.* 2019 Jul 9;20(13). pii: E3361. doi: 10.3390/ijms20133361.

C. **M. Sztretye,** Z.Singlár, L. Szabó, Á. Angyal, N.Balogh, F.Vakilzadeh, P. Szentesi, B.Dienes and L.Csernoch. (2020a) Improved Tetanic Force and Mitochondrial Calcium Homeostasis by Astaxanthin Treatment in Mouse Skeletal Muscle. *Antioxidants* **2020a,** 9(2), 98; https://doi.org/10.3390/antiox9020098.

D. **M. Sztretye**, Z.Singlár, N. Balogh, G.Kis, P. Szentesi, A. Angyal, I. Balatoni, L.Csernoch and B. Dienes (2020b) The role of Orai1 in regulating sarcoplasmic calcium release, mitochondrial morphology and function in myostatin defficient skeletal muscle, *Frontiers in Physiology*, **2020b.** Dec 21;11:601090. i: 10.3389/fphys.2020.601090

E. **M. Sztretye**, Szabó L, Dobrosi N, Fodor J, Szentesi P, Almássy J, Magyar ZÉ, Dienes B, Csernoch L. (2020c) From mice to humans: an overview of the potentials and limitations of current transgenic mouse models of major muscular dystrophies and congenital myopathies.. *Int J Mol Sc*i. **2020c** Nov 25;21(23):8935. doi: 10.3390/ijms21238935.

F. Z. Singlár, P. Szentesi, J. Fodor, Á. Angyal, L. Csernoch, **M. Sztretye:** Assessing the Potential of Nutraceuticals as Geroprotectors on Muscle Performance and Cognition in Aging Mice. *Antioxidants* 2021, 10, 1415. https://doi.org/10.3390/antiox10091415

G. M. Gönczi; A.Csemer; L. Szabó; **M. Sztretye**; J. Fodor; K. Pocsai; K.Szenthe; A.Keller-Pintér; Z.Márton Köhler; P. Nánási; N.Szentandrássy; B. Pal; L. Csernoch. Astaxanthin exerts anabolic actions via pleiotropic modulation of the excitable tissue" *CMLS* 2021-*submitted*

Other:

Zoltán Singlár, have been involved in the present research initially as an undergraduate student participant. He attended the Scientific Student Affair Conference at the University of Debrecen, held between 2019.02.13-2019.02.15 where he was ranked 3rd place. In May 2019, he successfully got his MSc diploma and in September 2019 he was enrolled in the doctoral program of the Doctoral School of Molecular Medicine (MODI) under my supervision. In 2021, he was awarded the ÚNKP scholarship.

In September 2021 two new PhD students (Szilagyi Catalin Vlad and Nyamkhuu Ganbat via Stipendium Hungaricum fellowship) were enrolled in the Doctoral School of Molecular Medicine (MODI) under my supervision. Three undergraduate students have also been participating in the research work.

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