Autophagy role in Glioblastoma Oncobiology

Gargano D.[1], Colardo M.[1], Colella B.[1], Lopa C.[1] Santoro G.[1] and Di Bartolomeo S.[1]

1Dept. of Biosciences and Territory, University of Molise, Pesche (IS), Italy

Background

Autophagy is an highly conserved homeostatic process which degrades and recycles intracellular components both normally and expecially under stressful conditions[1]. The role of autophagy in tumour onset and progression is debated with a preventive or promoting function during early or advanced stage of cancerogenesis respectively[2]. In the context of gliomagenesis autophagy also plays a paradoxical role as can promote or suppress GBM. However, in recent years, a growing amount of data suggests a progressive decrease of autophagy competence during glioma initiation[3-4].

Aim & Methods

Preliminary databanks analysis has shown that ULK1, but not ULK2, transcript is downregulated in GBM compared to normal brain, suggesting an alteration of the autophagy process (Fig.1). In order to assess the autophagy competence of GBM we are investigating the expression levels of the main autophagy players by Western blotting and Immunohistochemistry analysis. Moreover, molecular mechanisms of ULK1 regulation will be investigated by transcriptomic and proteomic analysis.

Results

ULK1 Transcriptomic analysis in GBM

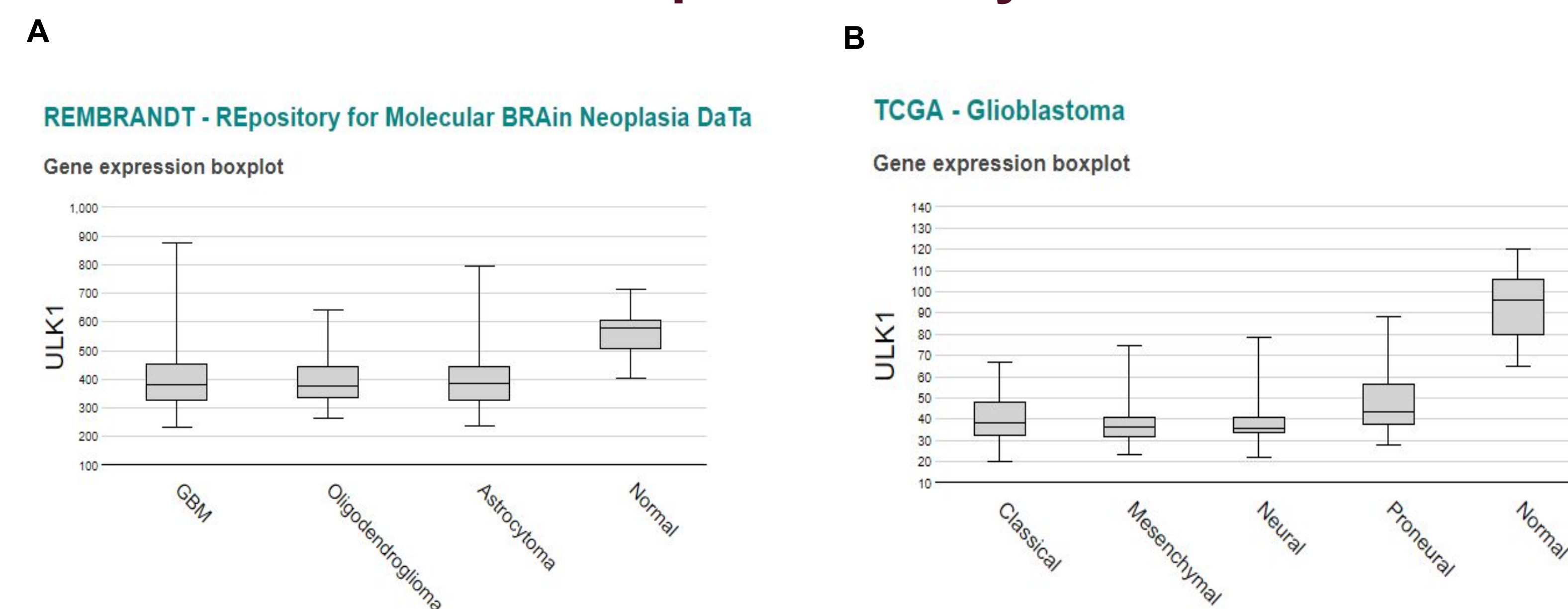

Fig.1 (**A**) Boxplot of ULK1 mRNA in normal brain tissue and in the indicated kind of glioma, based on analysis od REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT). (**B**) Boxplot of of ULK1 mRNA in the four GBM subtypes indicated, compared to normal brain tissue, based on The Cancer Genome Atlas (TCGA) data.

Autophagy status in GBM

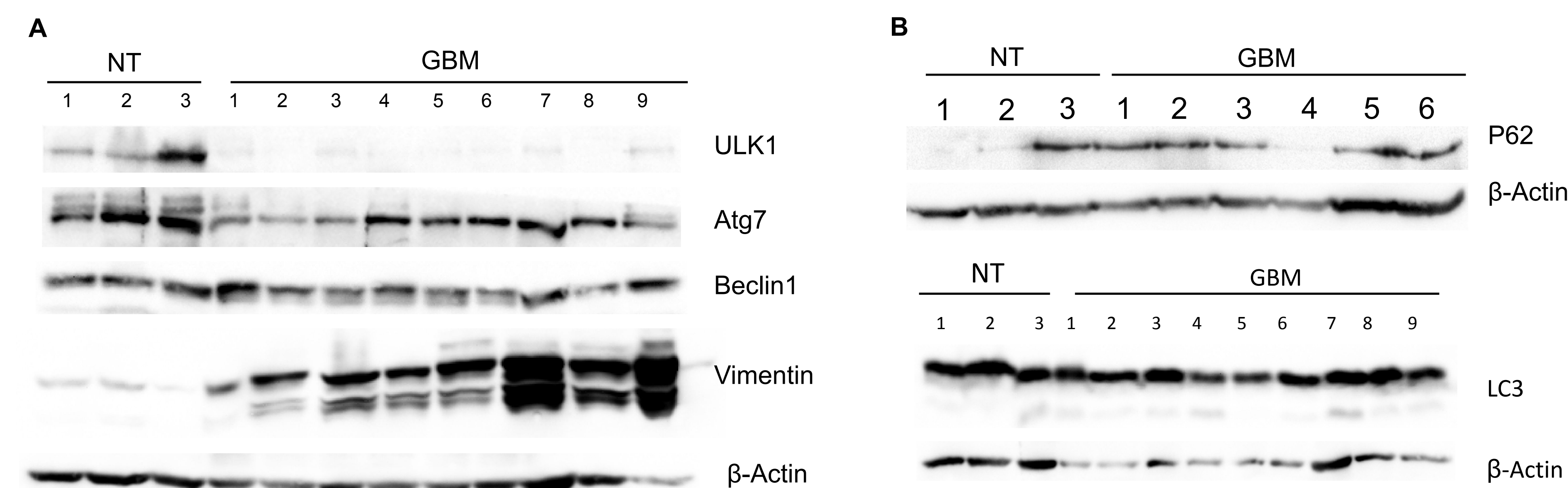

Fig.2 Protein extracts from brain non-tumoral (NT) and GBM surgical resections were analyzed by western blotting analysis using specific antibodies. (**A**) Expression of ULK1, Atg7, Beclin1 was assessed. (**B**) The autophagic flux was analyzed testing degradation of autophagy substrate p62 and LC3I in LC3 II conversion. Vimentin expression was used as marker of aggressiveness and β-Actin as loading control.

ULK1 protein expression in GBM

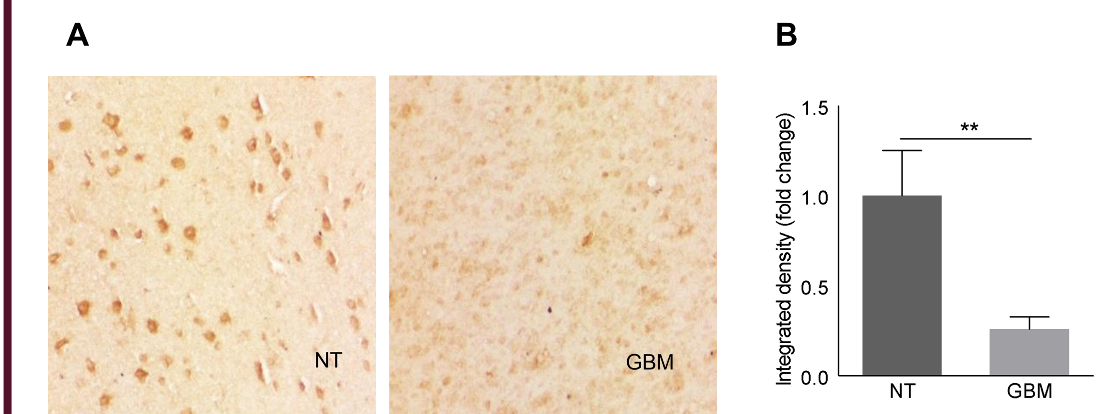

Fig.3 (**A**) Immunohistochemical analysis of ULK1 in fixed and embedded biopsies of GBM and non-tumoral (NT) brains is shown. (**B**) The graph represents the mean integrated density ± SD of three different pictures/sample. Statistical significance: ** $p < 0.01$ Student t-test

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Conclusion

Our data support the hypothesis of an autophagy decreased proficiency within the tumoral mass likely due to a negative modulation of the autophagy initiator ULK1. However, further in vitro and in vivo analyses are needed to assign a defined role of autophagy during gliomagenesis.

d.gargano@studenti.unimol.it

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Characterization of a Zebrafish setd5 Loss-of-Function model: Insights into neurodevelopmental and behavioural deficits associated with ASD

Matteo Digregorio[1], C. De Cesari[1], C. Gabellini[1], C. Pucci[1,2], D. Martini[1], R. Mosca[1], William Norton[3], Vania Broccoli[4,5], Alessandro Sessa[4], Massimiliano Andreazzoli[1]

[1]Department of Biology, University of Pisa, Italy, [2]Sant'Anna School of Advanced Studies, Pisa, Italy, [3]Department of Neuroscience, Psychology and Behaviour, University of Leicester, UK, [4]Stem Cell and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy, [5]CNR Institute of Neuroscience, Milan, Italy

Background

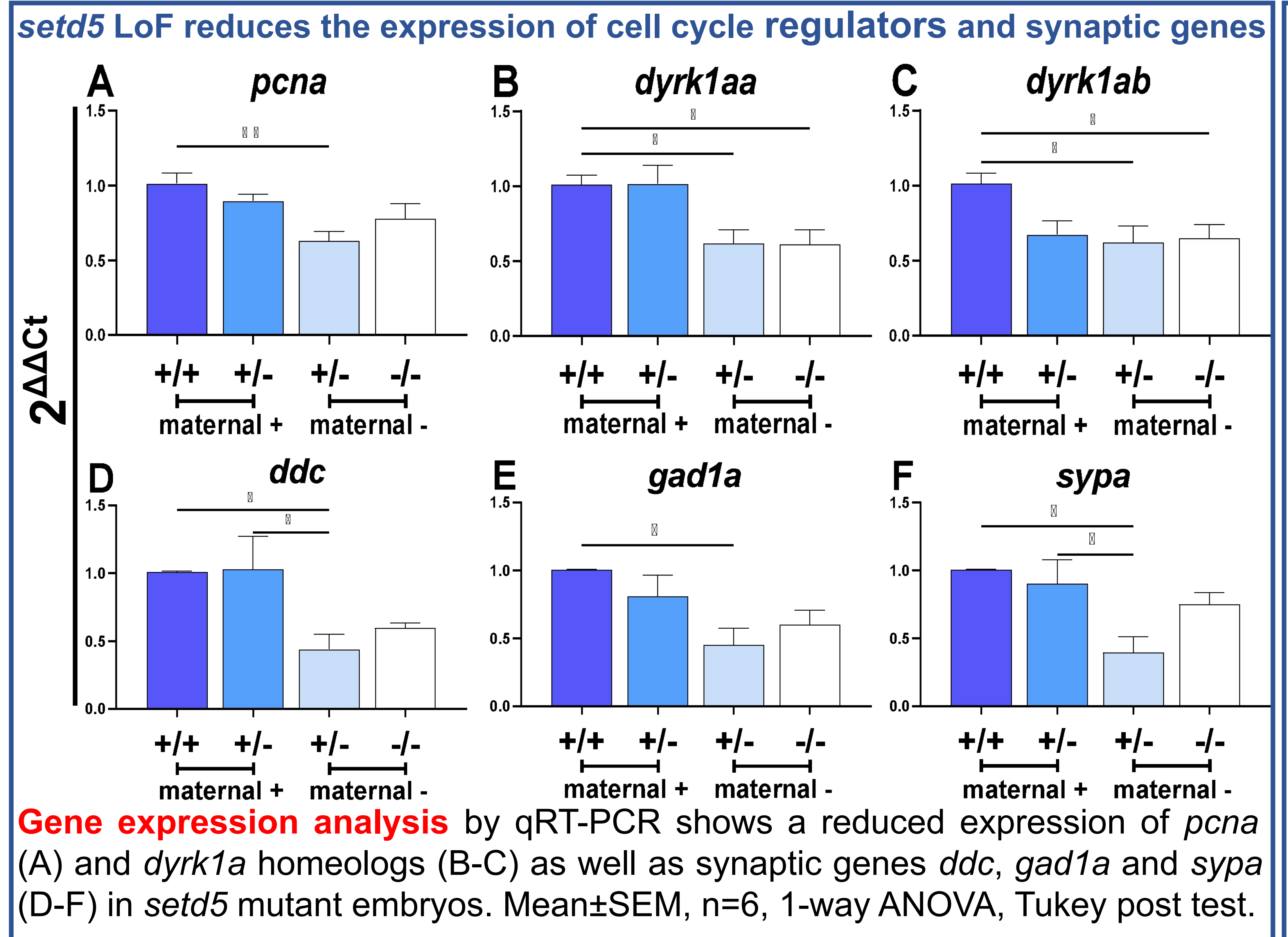
SETD5 LoF mutations are linked to intellectual disability and autistic spectrum disorders in humans, along with development defects[1]. *SETD5* encodes a histone methyltransferase that is highly expressed in the brain, and its haploinsufficiency leads to reduced methylation of Histone 3 Lysine 36 (H3K36). SETD5 protein sequence is conserved between zebrafish and humans, and our preliminary data showed that *setd5* mRNA is maternally expressed during early embryo development and that mutants exhibit cranial alterations, including microcephaly[2,3].

Aim & Methods

The aim of this study is to investigate the role of *setd5* in neurodevelopment and behavioural outcomes using a zebrafish mutant model. We aim to characterize molecular and cellular mechanisms underlying neurodevelopmental defects in *setd5* mutant embryos, and understand the associated social and memory. By studying setd5 deficiency, we aim to contribute to our understanding of developmental disorders and identify potential therapeutic targets.

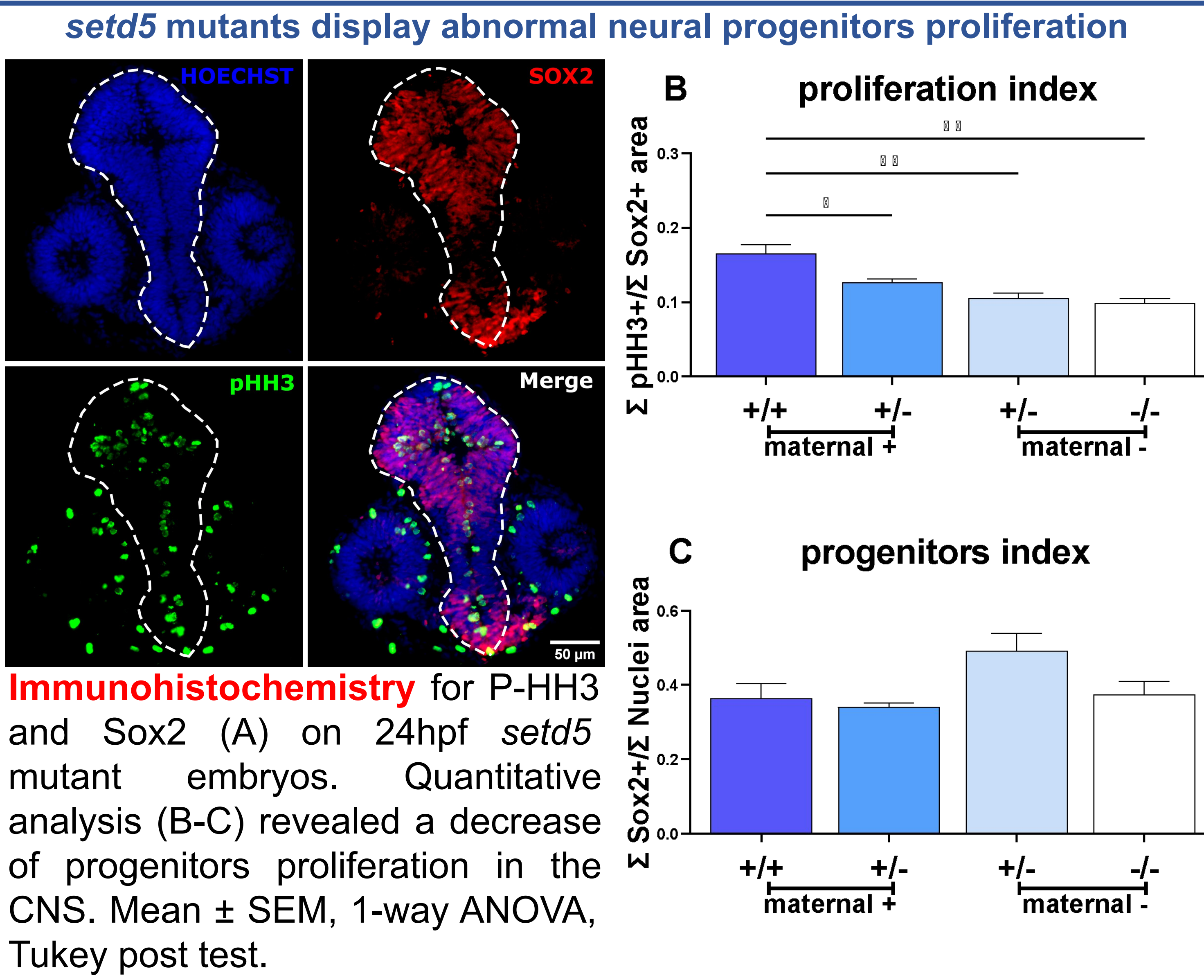
Results

setd5 LoF reduces the expression of cell cycle regulators and synaptic genes



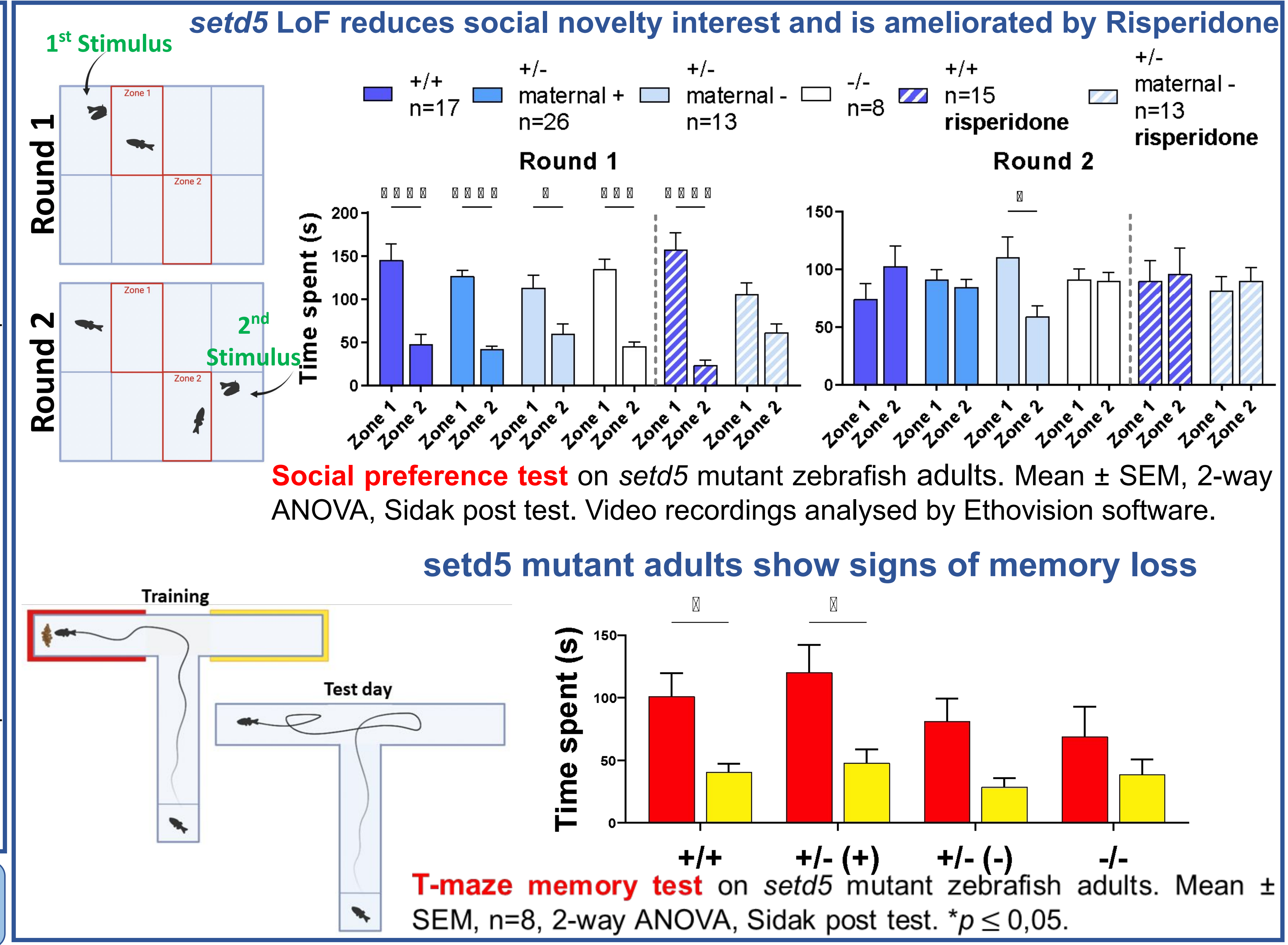
Gene expression analysis by qRT-PCR shows a reduced expression of *pcna* (A) and *dyrk1a* homeologs (B-C) as well as synaptic genes *ddc*, *gad1a* and *sypa* (D-F) in *setd5* mutant embryos. Mean±SEM, n=6, 1-way ANOVA, Tukey post test.

setd5 mutants display abnormal neural progenitors proliferation



Immunohistochemistry for P-HH3 and Sox2 (A) on 24hpf *setd5* mutant embryos. Quantitative analysis (B-C) revealed a decrease of progenitors proliferation in the CNS. Mean ± SEM, 1-way ANOVA, Tukey post test.

setd5 LoF reduces social novelty interest and is ameliorated by Risperidone



Social preference test on *setd5* mutant zebrafish adults. Mean ± SEM, 2-way ANOVA, Sidak post test. Video recordings analysed by Ethovision software.

setd5 mutant adults show signs of memory loss

T-maze memory test on *setd5* mutant zebrafish adults. Mean ± SEM, n=8, 2-way ANOVA, Sidak post test. $^{*}p \leq 0{,}05$.

Conclusion

Our *setd5* mutant zebrafish model exhibits neurodevelopmental alterations, decreased expression of genes for synaptic proteins and cell cycle regulators. Along with the behaviour and social impairments we described, these findings emphasize the importance of *setd5* in CNS function. Additionally, the presence of *setd5* maternal mRNA during early embryo development significantly impacts neurological outcomes. Taken together, our data show that the *setd5* mutant model could be a valuable tool to explore potential pharmacological treatments od *SETD5* haploinsufficiency.

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matteo.digregorio@phd.unipi.it

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HIGH-FAT DIET INDUCES CELL DAMAGE IN RAT EPIDIDYMIS

Università degli Studi della Campania Luigi Vanvitelli

D. Latino[1], S. Falvo[1], A. Santillo[1], G. Chieffi Baccari[1], M.M. Di Fiore[1]

[1]University of Campania "Luigi Vanvitelli",
Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Caserta, Italy

Dipartimento di Scienze e Tecnologie Ambientali Biologiche e Farmaceutiche

Background

The epididymis plays an essential role in reproduction by promoting sperm maturation. It is divided into three main anatomical segments called the caput, corpus and cauda. The main function of the initial segments, the caput and corpus, is to provide a luminal environment suitable for sperm maturation, while the cauda is responsible for the storage of the mature spermatozoa. It has been widely reported that a high-fat diet (HFD) impairs reproductive performance by causing testicular dysfunction. Other studies have suggested a causative role of HFD in oxidative stress, which is the main cause of damage to reproductive function.

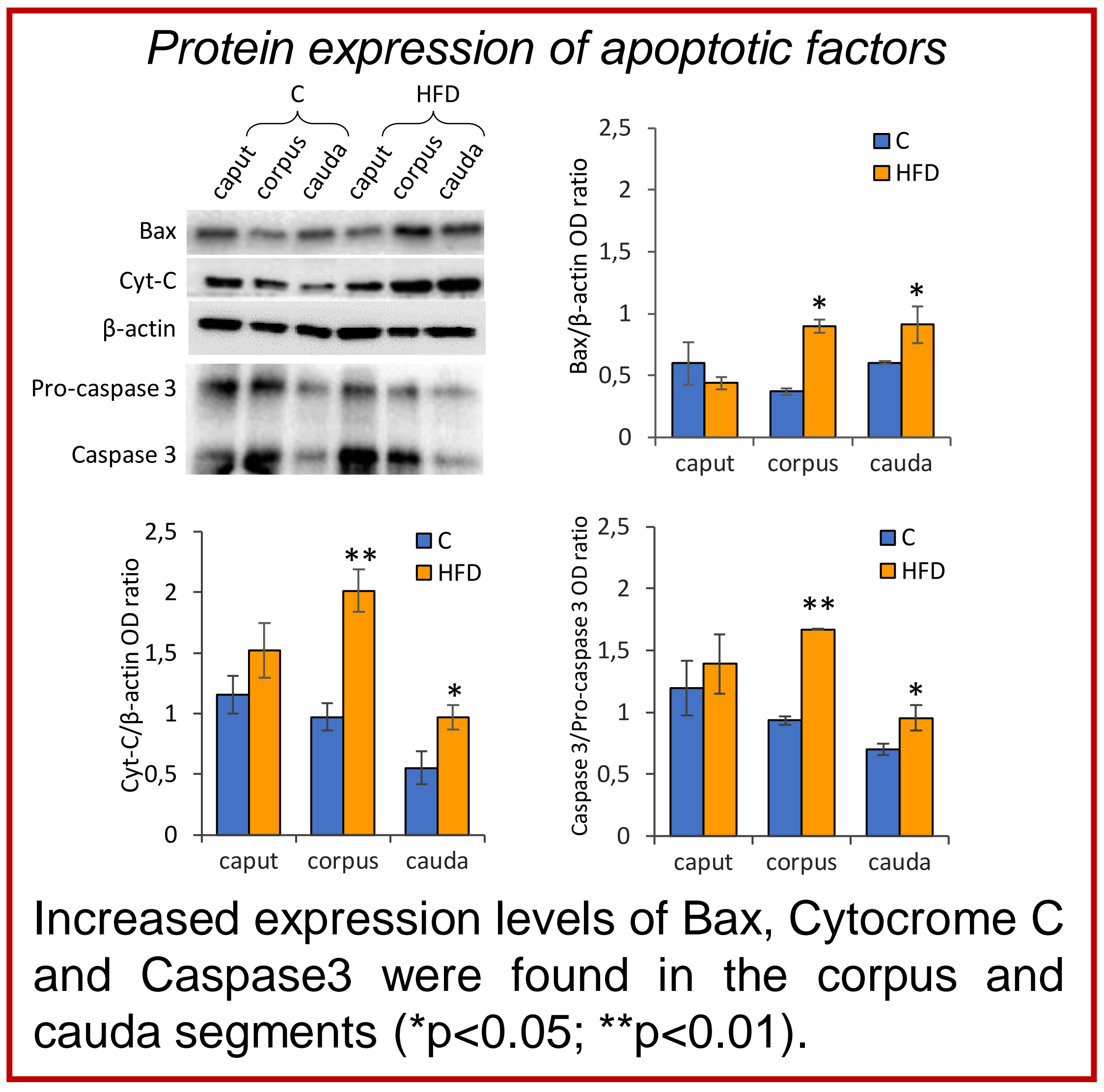
Aim & Methods

In order to study the response induced by lipid overload in the rat epididymis, the animals were housed under thermoneutral conditions (28-30°C) in conjunction with an HFD. We investigated the cellular response to HFD in the three regions of the rat epididymis, first by assessment of the oxidative state. We also determined the effects of HFD on autophagy/selective autophagy (lipophagy), apoptosis, and proliferation. Expression levels of 1) antioxidant enzyme, 2) apoptosis-related factors, 3) proliferation marker, and 4) autophagy and selective autophagy-related factors were assessed by Western blot analysis.

Results

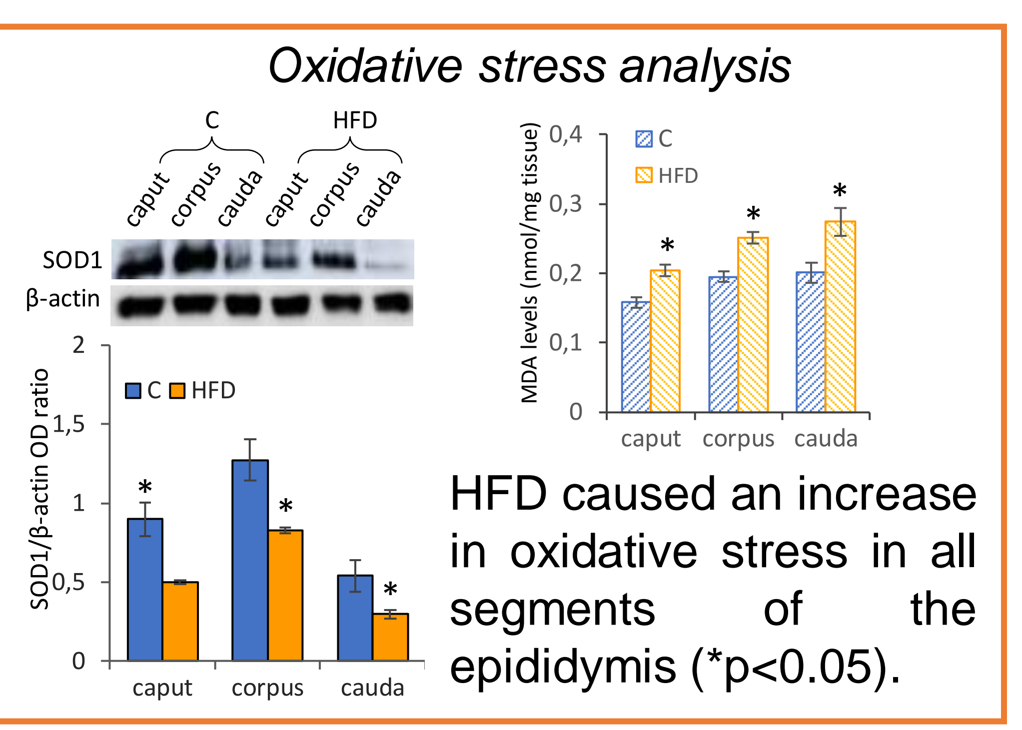
	C	HFD
Body weight (g)	377 ± 9.23	450 ± 6.09*
Food Intake (g)	37.9 ± 0.919	42.2 ± 1.06*
Respiratory quotient (QR)	0.92 ± 0.01	0.79 ± 0.005*
Cholesterol (mg/dl)	50 ± 3.03	82.30 ± 3.16*
Triglycerides (mg/dl)	145 ± 11.77	287.36 ± 38.29*

Protein expression of apoptotic factors



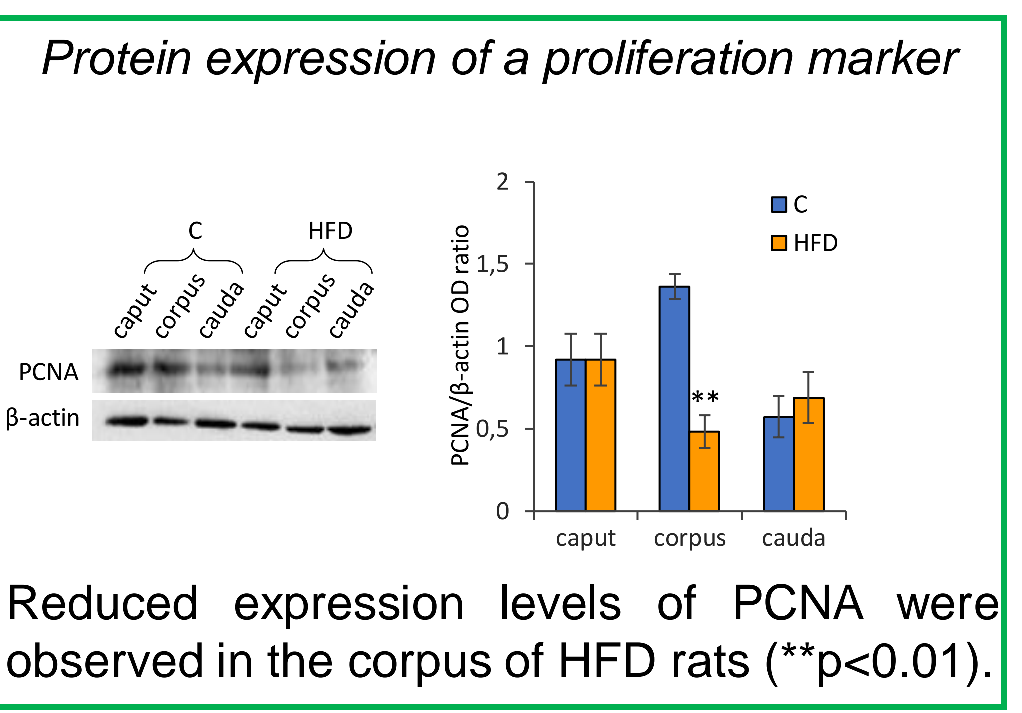
Increased expression levels of Bax, Cytocrome C and Caspase3 were found in the corpus and cauda segments (*p<0.05; **p<0.01).

Oxidative stress analysis



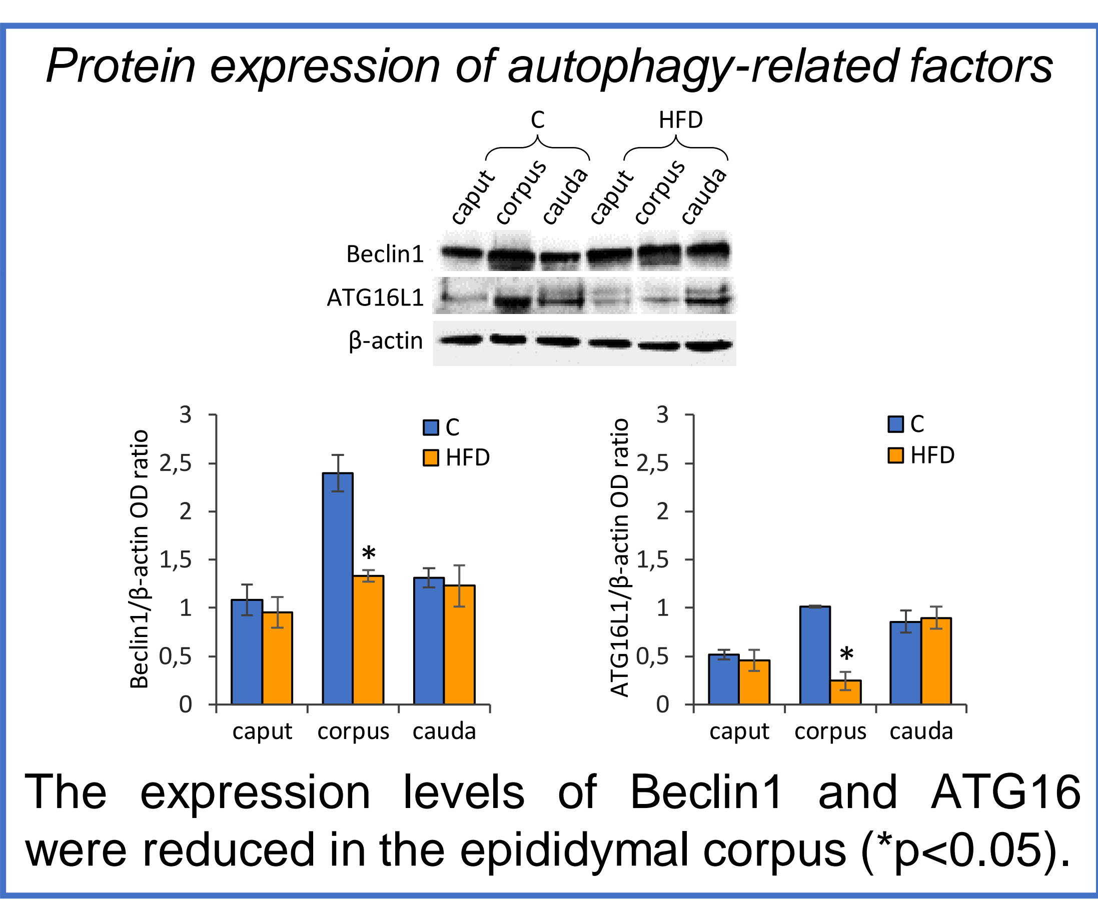
HFD caused an increase in oxidative stress in all segments of the epididymis (*p<0.05).

Protein expression of a proliferation marker



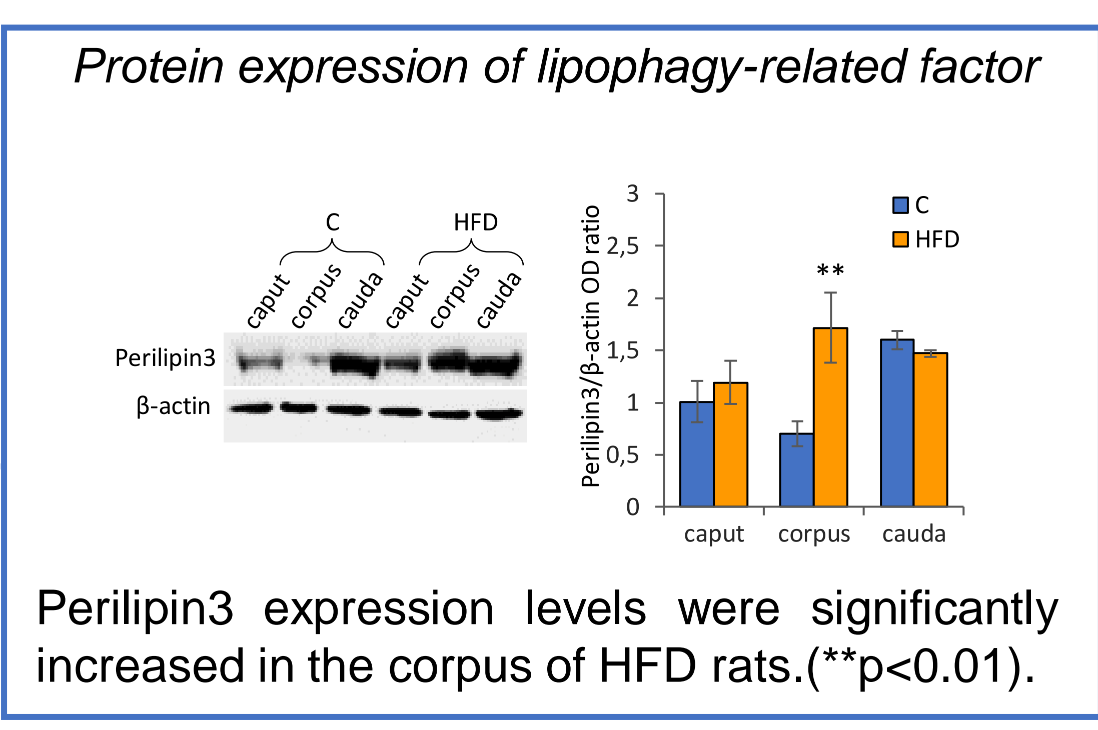
Reduced expression levels of PCNA were observed in the corpus of HFD rats (**p<0.01).

Protein expression of autophagy-related factors



The expression levels of Beclin1 and ATG16 were reduced in the epididymal corpus (*p<0.05).

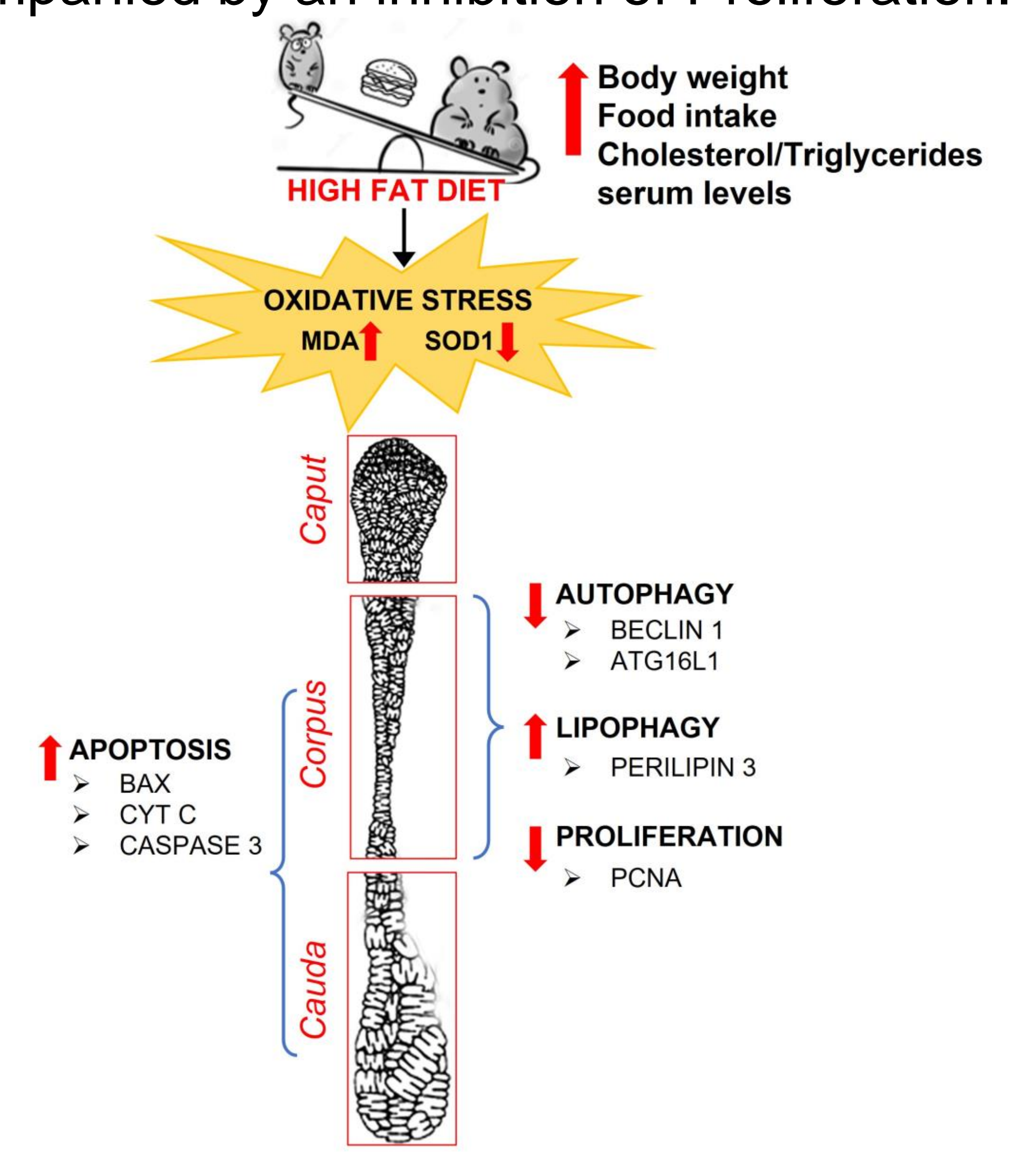
Protein expression of lipophagy-related factor



Perilipin3 expression levels were significantly increased in the corpus of HFD rats.(**p<0.01).

Conclusion

The oxidative stress, caused by HFD, influenced different cellular response mechanisms, mostly detected in the corpus and cauda regions. Lipophagy occurred as a protective response to lipid accumulation; decreased Autophagy suggested an inability of epididymal cells to counteract oxidative stress and maintain homeostasis; increased Apoptosis occurred to eliminate dysfunctional cells and it was accompanied by an inhibition of Proliferation.



debora.latino@unicampania.it

BLOOD TESTIS BARRIER PROTEINS IN AUTISM MODEL MOUSE

68° Convegno GEI - SIBSC Messina 2023

M. Prisco, S. Valiante, T. Chianese, T. Barra, G. Trinchese, M.P. Mollica, L. Rosati

University of Naples Federico II, Department of Biology, Naples, Italy

e-mail: marina.prisco@unina.it

Background

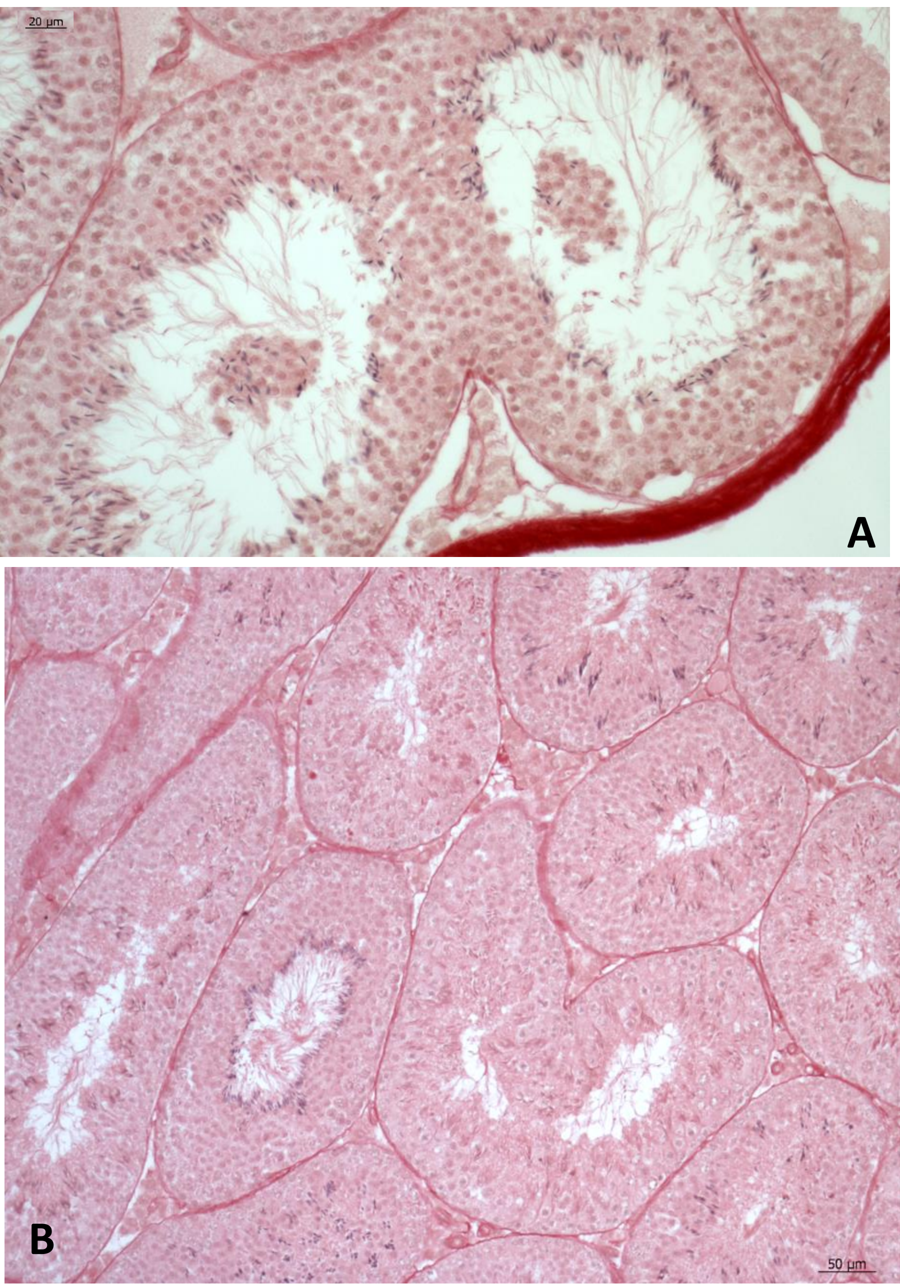
Autism spectrum disorders (ASD) are neurodevelopmental diseases with complex symptoms but neurobiological basis poorly understood to this day. Pathogenesis seems to be linked to combination of genetic, autoimmune, environmental, and perhaps in utero risk factors leading to neuroinflammation [1]. Alterations of the blood brain barrier and of intestinal epithelial barrier, due to high levels of inflammation and to changes in tight junctions (TJ) protein expression [2] are characteristic in human ASD: inappropriate antigen trafficking through impaired barriers, followed by inflammation can be part of the chain of events leading to these disorders. To the best of our knowledge, there are no studies on the blood testis barrier.

Aim & Methods [size: 28 p.]

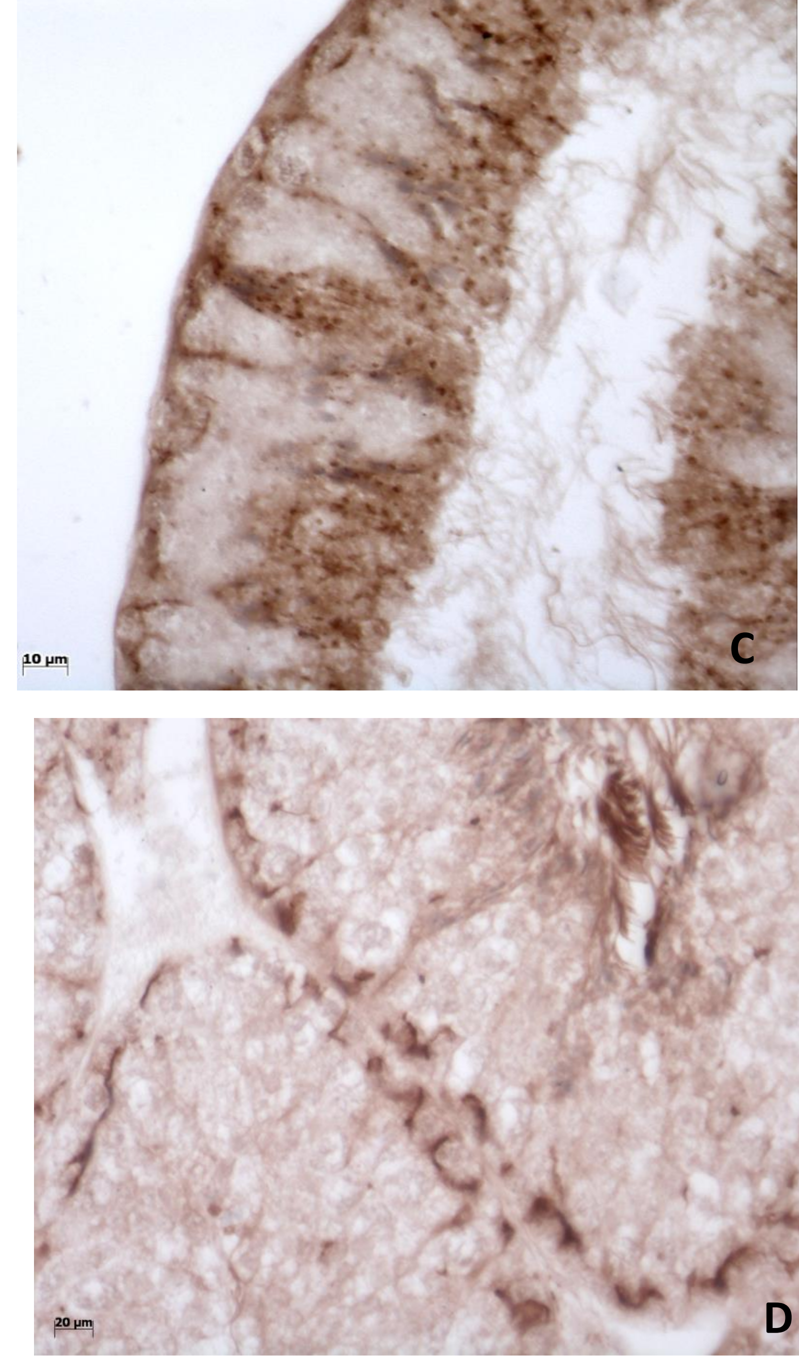
We starting to study the testis of BTBR T+tf/J mouse, a well-validated model of idiopathic autism: we carried out investigations on testis structure and on expression of two proteins, claudin-5, a tight junctions protein, and connexin 43, a junctional complex protein.

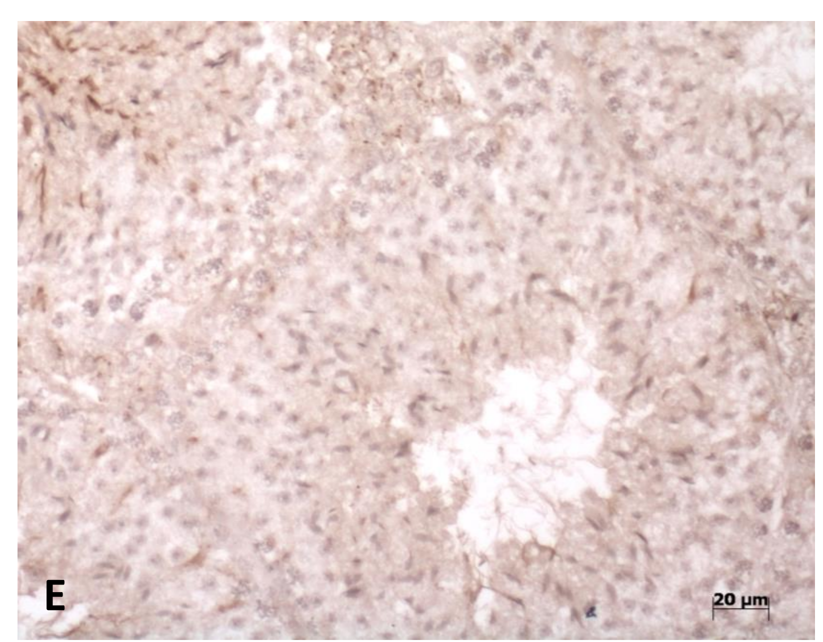
We performed histological investigation and immunohistochemistry technique on testis of wild-type animals, BTBR T+tf/J mouse and BTBR mouse (named BTBR+D) treated with a mix of dimethylglycine and B group vitamins, with know anti-inflammatory activity.

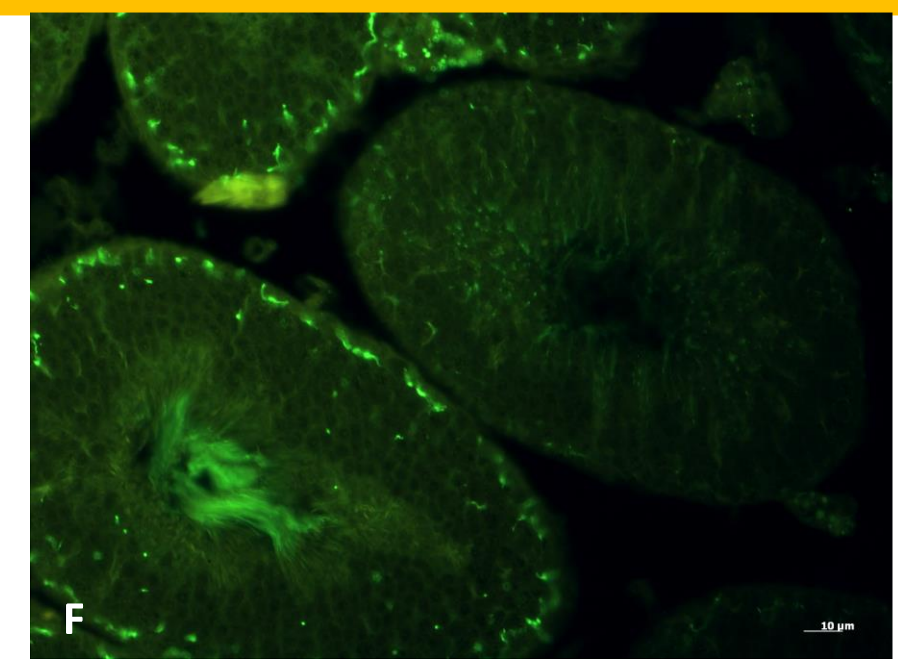
Results

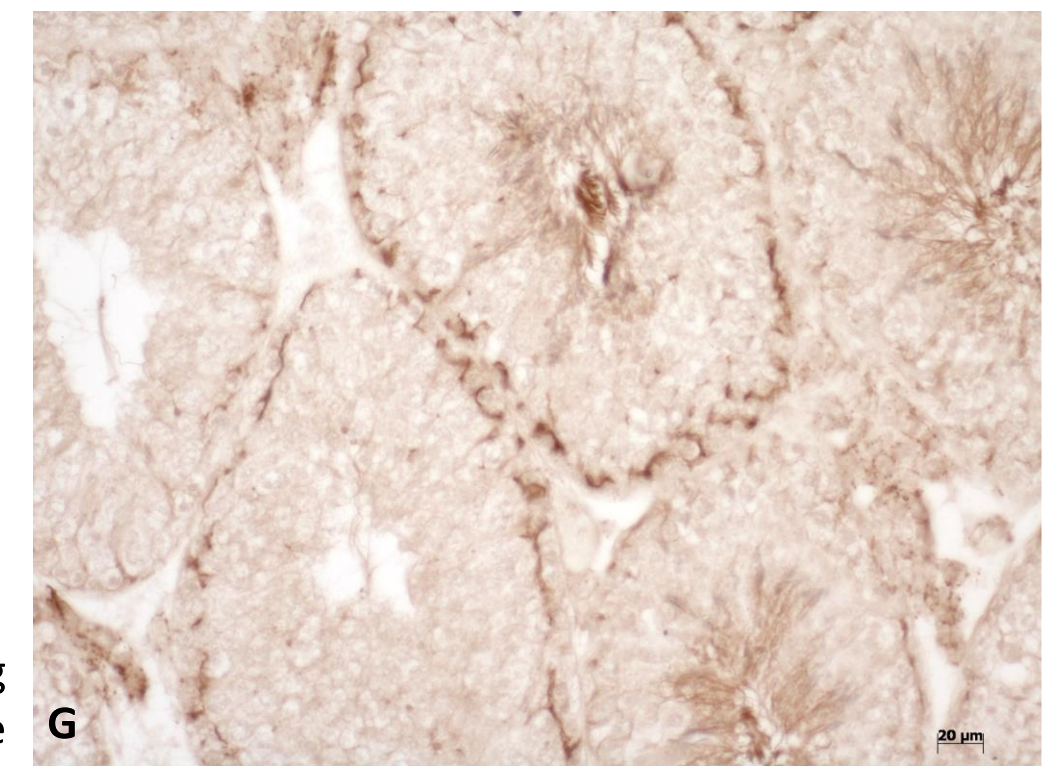


In BTBR mouse testis clusters of immature cells were observed in the lumen of many seminiferous tubules, together with or instead of spermatozoa (A); in BTBR+D the morphology of most of the tubules appears normal (B).









Wild-type testis showing intense claudin-5 (C) and connexin 43 (D) staining surrounding germ and Sertoli cells in all seminiferous tubules. In BTBR mouse the labelling decreases or disappears entirely (E), but it is partially recovered in BTBR+D samples (F and G).

Conclusion

In BTBR mouse the seminiferous tubules structure appears partially modified with evident areas of detachment between the cells, compared to the wild-type and the claudin5 and connexin 43 expression decreases. Treatment with a mix of dimethylglycine and B group vitamins, with anti-inflammatory action, seems to partially restore the tubule morphology, as already seen with vitamin B-12 supplementation [3, 4].

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68° Convegno GEI - SIBSC Messina 2023

POTENTIAL COCKTAIL EFFECT OF NONYLPHENOL AND SEX HORMONES ON HUMAN PROSTATE CELLS

A.Mileo[1] , F. Carrella[1] , S. Boccia[1] , R. Cominale[1] , M. De Falco[1,2]

[1]Department of Biology, University of Naples "Federico II", Naples; Italy

[2]National Institute of Biostructures and Biosystems (INBB), Rome, Italy.

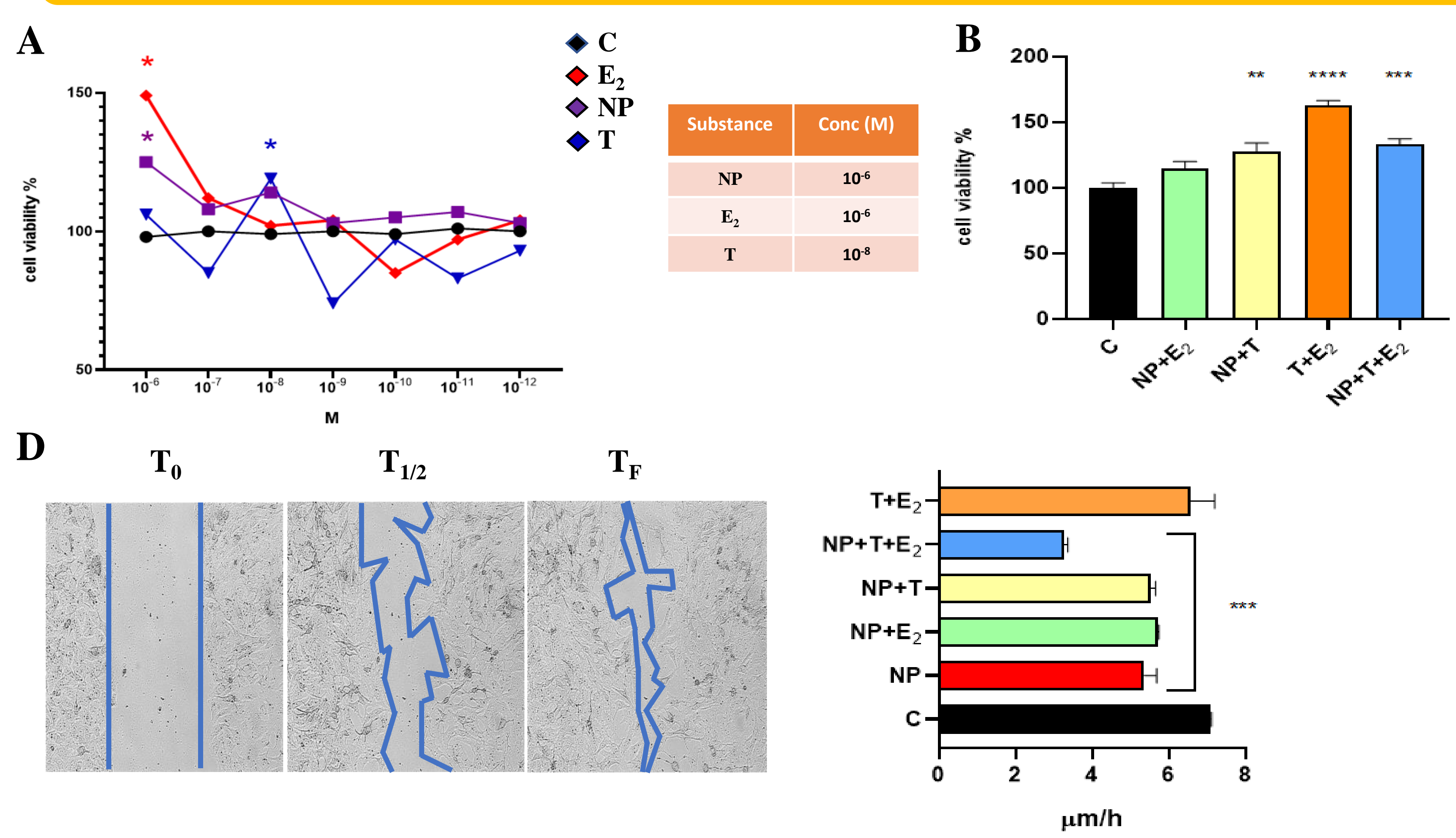
BACKGROUND

Nonylphenol (NP) belongs to a group of Endocrine disruptor chemicals (EDCs). It usually used in the manufacture of domestic, industrial, agricultural ~~products~~ and personal-care products, to improve their properties, as flexibility, durability, and transparency. It persists in different environmental matrices for a long time. So, it can bioaccumulate in adipose tissue and biomagnificate in food chain. Human population can mainly be exposed to EDCs, through ingestion or skin contact. Thanks to estrogen like behaviour, NP can interfere with human endocrine system, through estrogen receptors (ERs) pathways. Although NP has been extensively studied both *in vivo* and *in vitro*, it remains unclear if NP can interact with the sex hormone pathways, breaking the delicate ~~sex~~ hormonal balance.

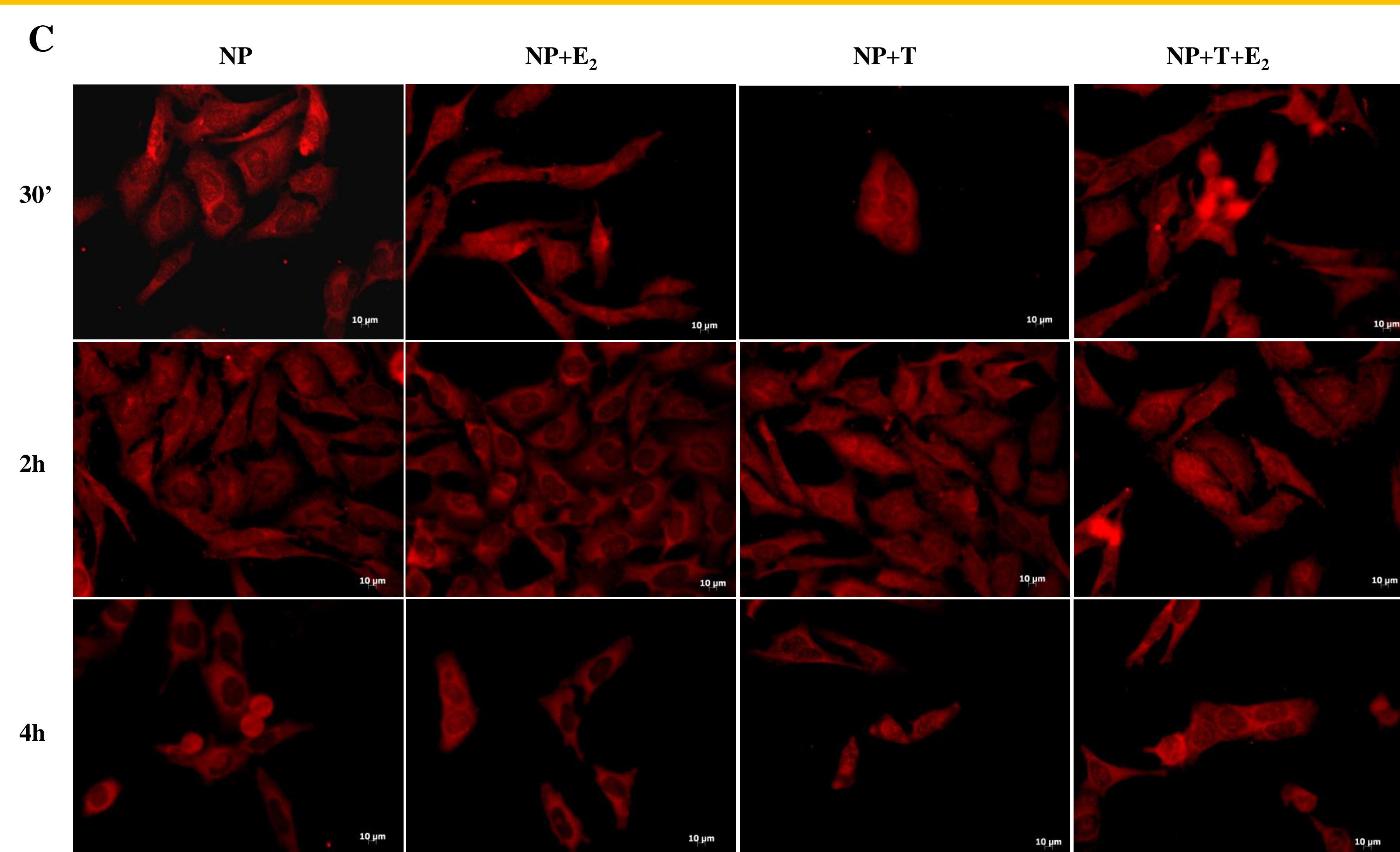
AIM & METHODS

The aim of this study is to deepen the knowledge about the potential cocktail effect of NP with sex hormones on human non tumoral prostate cells (PNT1A). First of all, **MTT assay** was carried out in order to detect the significant NP, Testosterone (T), 17 β-estradiol (E_2) concentrations that affected the cell viability, alone and in mixture. **Immunofluorescensce assay,** performed at different treatment time (30', 2h, 4h), allowed us to show ERα cytoplasm nucleus translocation after treatments. Moreover, healing average rate was calculate by **Wound-healing assay**.

RESULTS



Substance	Conc (M)
NP	10^{-6}
E_2	10^{-6}
T	10^{-8}



The significant NP, T and E_2 concentrations that affected the cell viability were represented in the **Fig. A**. Based on these data, mixtures for subsequent treatments were created. All the mixtures affected the cell viability in positive manner especially T+E_2, NP+T and NP+T+E_2 (**Fig. B**). ERα translocation was activated, especially after NP+T+E_2 treatment: It occurred after all the time treatment, suggesting the possible cooperation between NP and E_2 in mixture (**Fig.C**). In addition, the average migration rate was altered as a result of the treatments: it seemed like that in presence of NP, the average migration was slower than the control (**Fig. D**). No additive effect between NP and sex hormones was observed in all experiments.

CONCLUSION

We have pointed attention on dangerousness of the mixtures: all of these induced cell proliferation. Although NP showed an estrogen-like behaviour, activating ERα pathways, this chemical did not show additive responses with E_2 but it probably cooperated with T. Moreover, our results suggest that these mixtures could interfere with the dynamic structure of the cell cytoskeleton, altering prostate physiology, especially in the early stages of organ development.

Aldo.mileo@unina.it

Metabolic reprogramming and modulation of microglia in amyotrophic lateral sclerosis

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Vitolo S.[1], Migliarini S.[1], Scaricamazza S.[2], Votta A.[1], Ferri A.[2], Pasqualetti M.[1] and Ferraro E.[1]

[1]Dept. of Biology, University of Pisa, Pisa, Italy;

[2]Division of Experimental Neuroscience, IRCCS Fondazione Santa Lucia, Rome, Italy

Università di Pisa

bio Dipartimento di Biologia

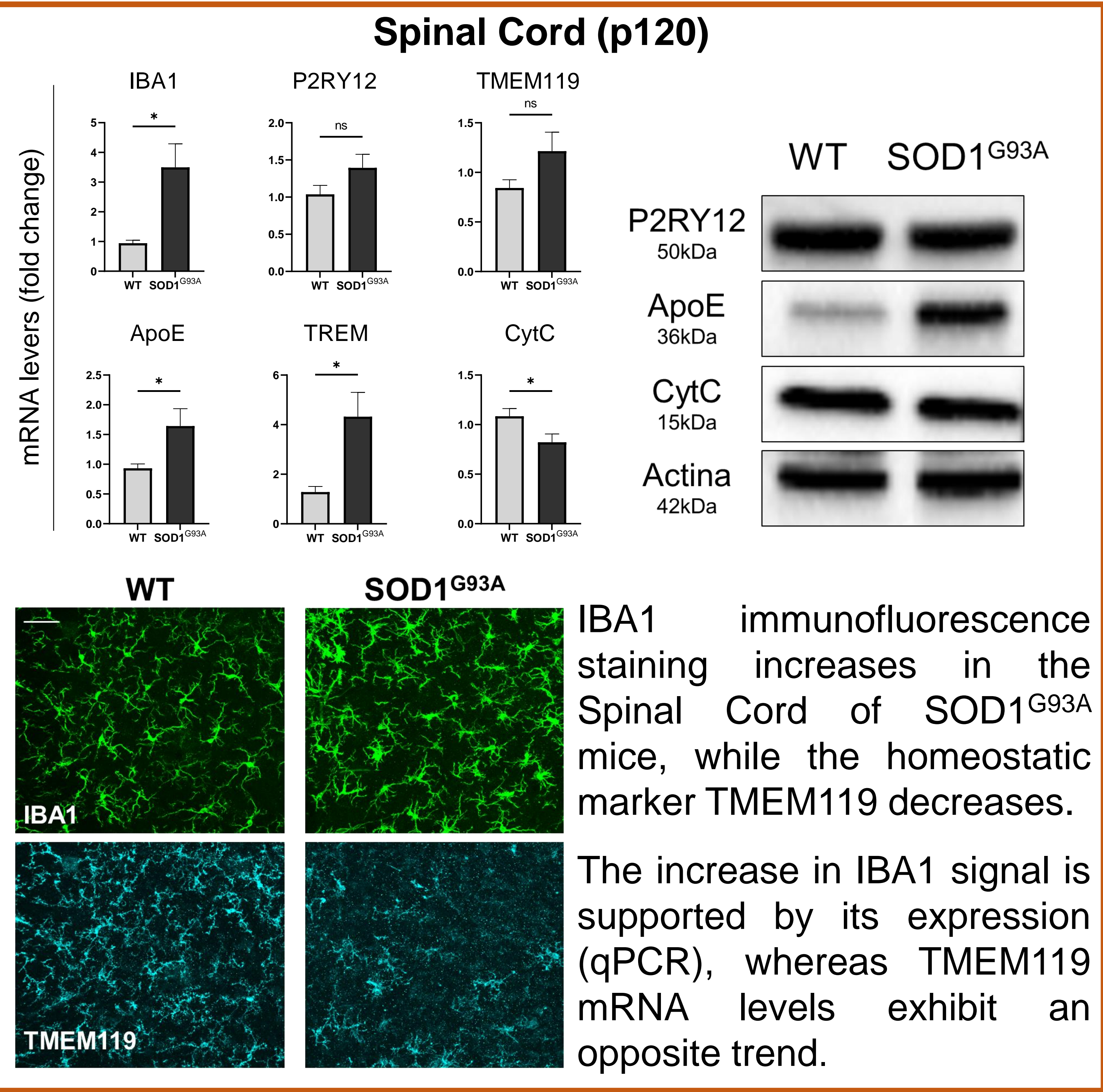
Background

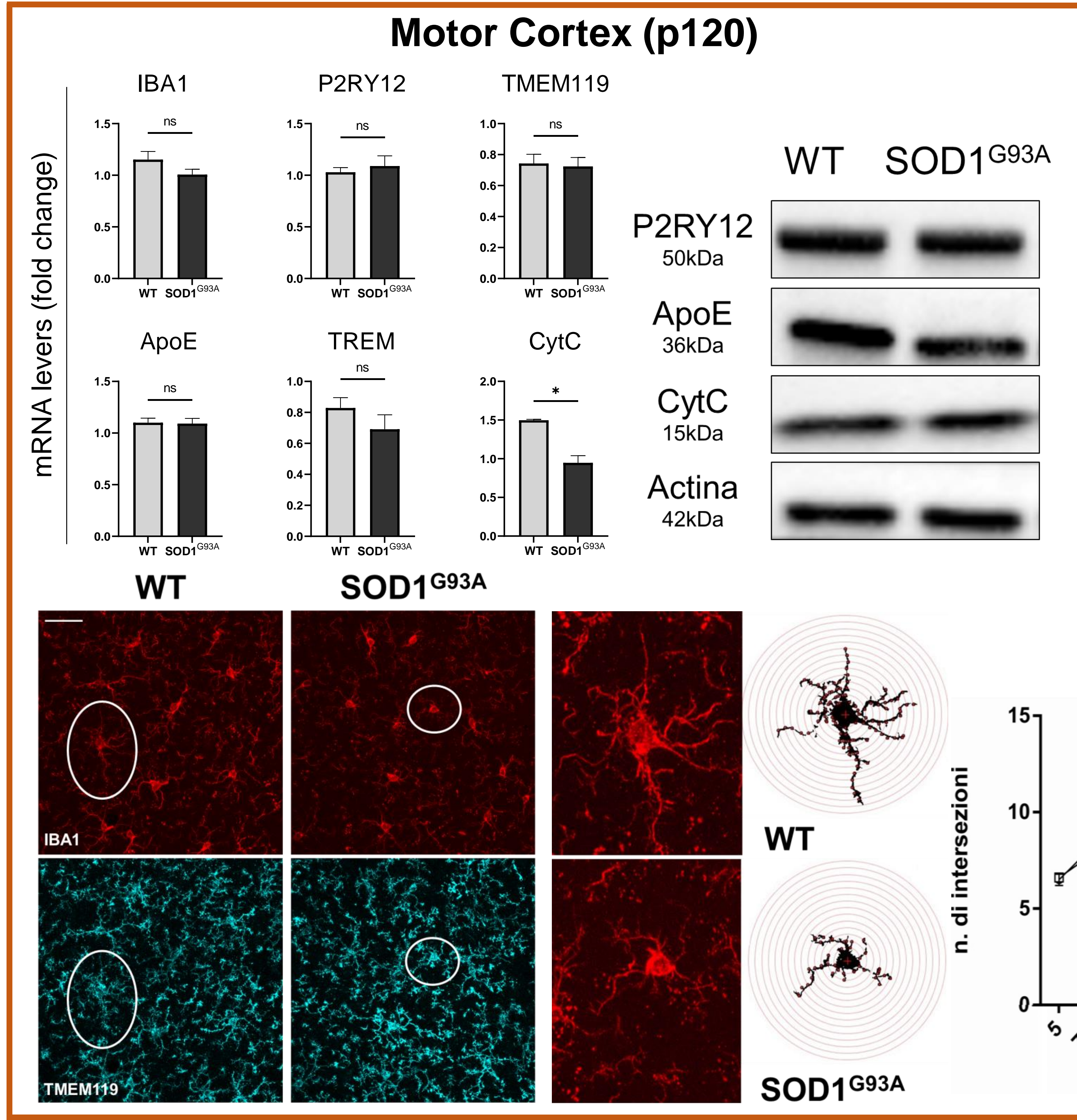
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the degeneration of upper and lower motor neurons. Motor neurons degeneration in ALS is associated with an inflammatory response that includes the activation of microglia. The activation of microglia has been demonstrated in the spinal cord, whereas its role in the encephalon it is still unclear[1,2].

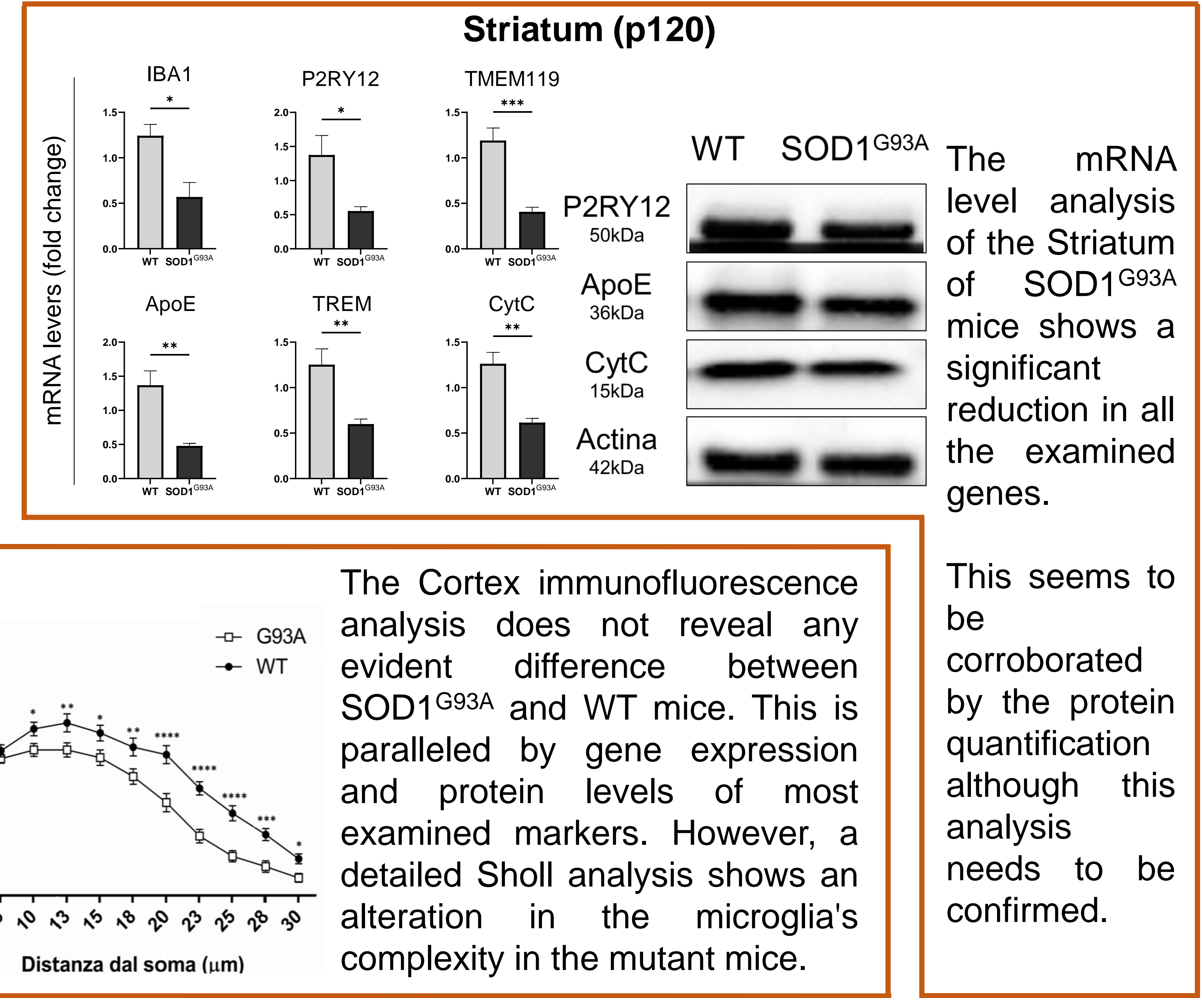
Aim & Methods

To investigate the role of cerebral microglia in this pathology, we have analysed the morphology of these cells and the expression of microglial molecular markers in the mouse model of ALS, SOD1^{G93A}. This analysis has been performed on different central nervous system (CNS) regions involved in the modulation of movement, and it has been compared with spinal cord microglia features.

Results







Conclusion

These data suggest that some brain aeras are highly affected in SOD1^{G93A}, although with different features compared to the spinal cord. A better understanding of the contribution of cerebral microglia in the pathogenesis of ALS is required.

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sara.vitolo2@phd.unipi.it

Effects of Trimetazidine on mitochondrial dysfunction in ALS SOD1^{G93A} cell models: an ultrastructural study

68° Convegno GEI - SIBSC Messina 2023

M. Muzzi[1,2], S. Moreno[1,2], I. Salvatori[3], S. Scaricamazza[3], A. Spalloni[3], C. Valle[3], A. Ferri[3]

[1]Dept. of Science, University Roma Tre, Rome, Italy; [2]Lab. of Neurodevelopmental Biology, Neurogenetics and Molecular Neurobiology and [3]Lab. of Neurochemistry, IRCCS Fondazione S. Lucia, Rome, Italy.

ROMA TRE UNIVERSITÀ DEGLI STUDI — SANTA LUCIA — SCIENZE Dipartimento di Eccellenza

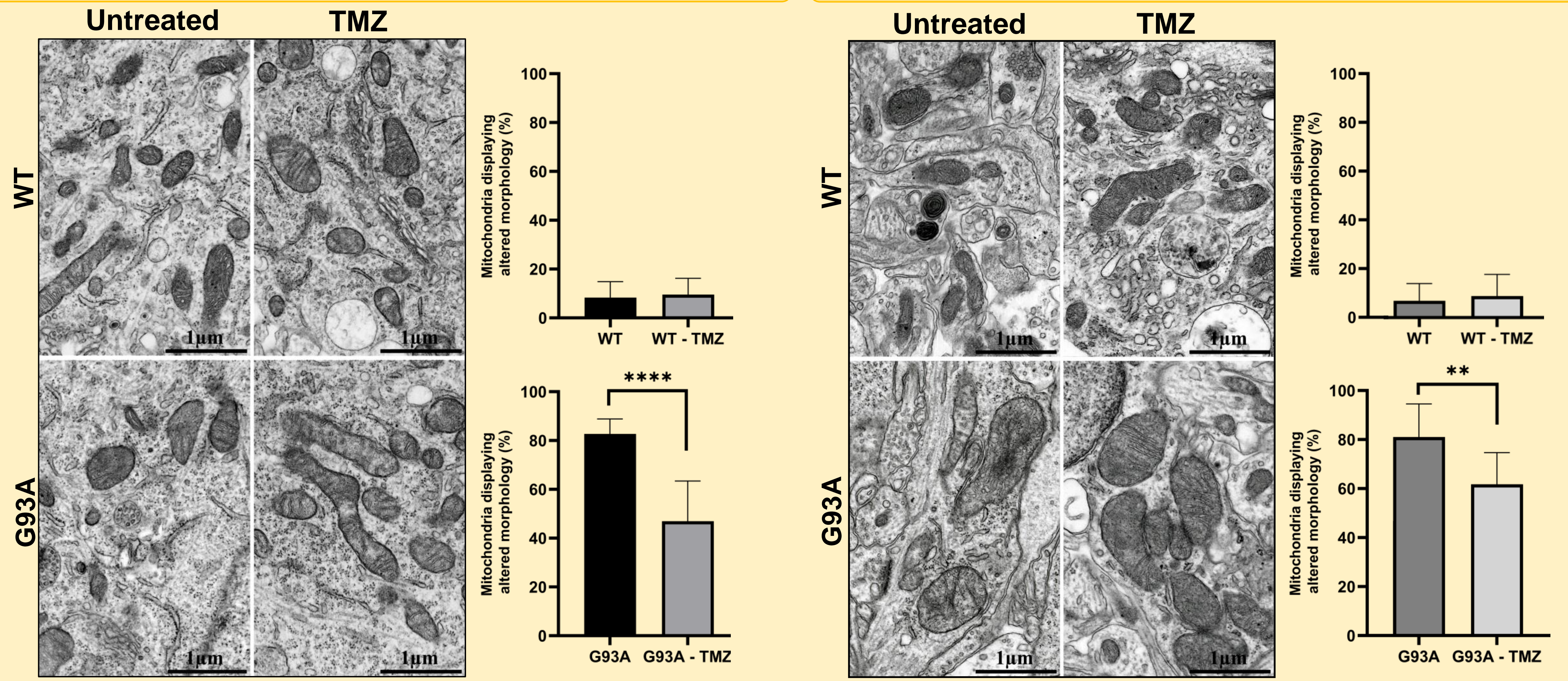
Background

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects upper motor neurons (MNs) of the cerebral cortex and lower MNs of the spinal cord, leading to progressive weakening of voluntary muscles. Several ultrastructural analyses[1] highlighted that, in both ALS patients and mouse models, MNs show mitochondria abnormalities, associated with disturbed mitophagy, mitochondriogenesis and calcium homeostasis[2].

Aim & Methods

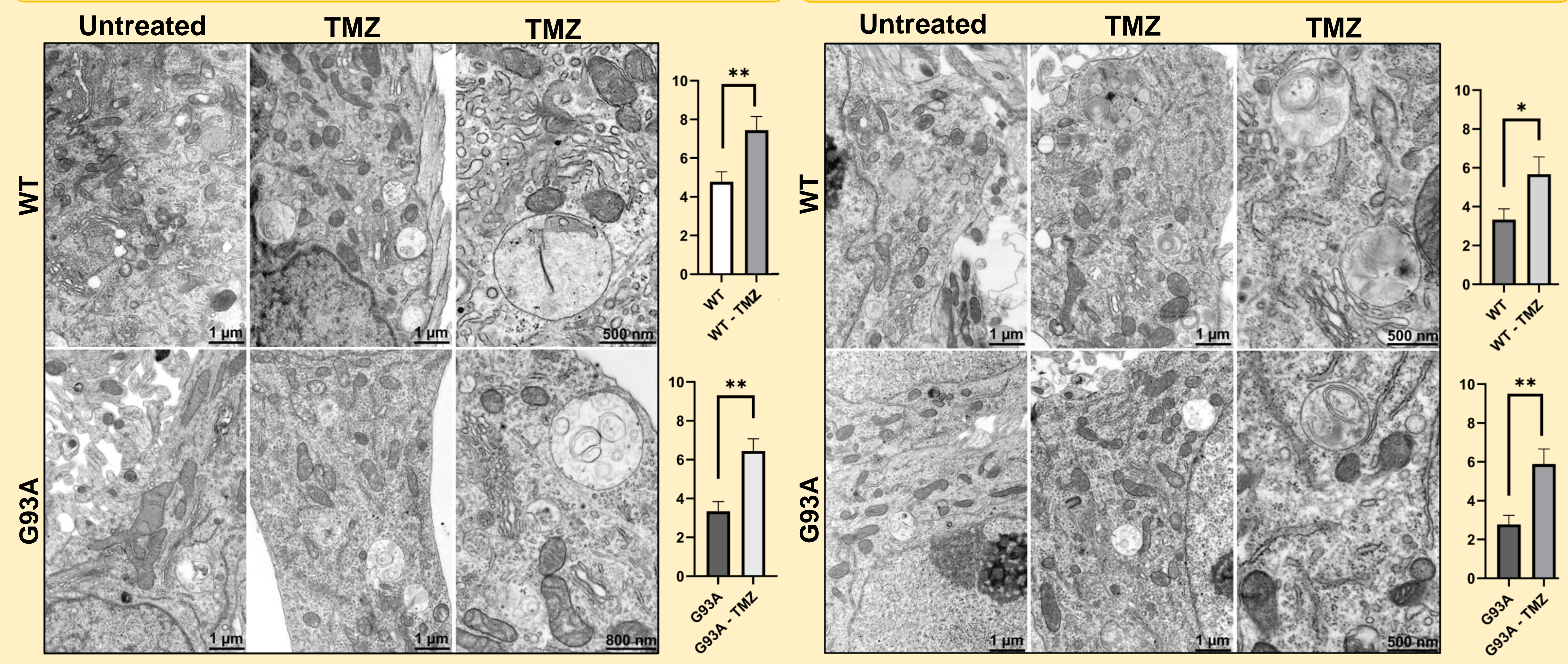
Trimetazidine (TMZ) is a metabolic modulator exerting protective effects on SOD1^{G93A} mice, preventing loss of spinal cord MNs and reducing neuroinflammation[3]. Even though the molecular mechanisms underlying its action are yet to be identified, TMZ certainly improves mitochondrial functionality. In the present study we investigated the effects of TMZ on mitochondrial ultrastructure, using primary cultures of cortical and spinal MNs obtained from embryos of SOD1^{G93A} mice and their wild type littermates.

Results: analysis of mitochondrial morphology



- ✓ WT cells displayed mitochondria with regular and parallel *cristae*, while SOD1^{G93A} cells exhibited remarkable alterations in the inner mitochondrial membranes. Specifically, *cristae* appeared poorly developed and misaligned in cortical cells, while *cristae* were strongly deranged and fragmented in spinal cells.
- ✓ In both cell cultures from SOD1^{G93A} mice, TMZ treatment resulted in a significant recovery of mitochondrial morphology. Conversely, following treatment, WT cell showed unchanged mitochondrial morphology.

Results: analysis of autophagosomes



- ✓ Following TMZ treatment, we detected a statistically significant increase in the number of autophagosomes, irrespective of cell type or even genotype.
- ✓ Autophagosomes displayed heterogeneous content, including recognizable mitochondria, suggesting that TMZ beneficial effects on mitochondrial functionality may at least partially relate to its ability to promote mitophagy.

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Acknowledgments

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maurizio.muzzi@uniroma3.it

68° Convegno GEI - SIBSC Messina 2023

MMP-2 and MMP-9 Expression Levels in Cerebrospinal Fluid and Derived-Extracellular Vesiscles from Patients with Multiple Sclerosis

UNIVERSITÀ DEGLI STUDI DI PALERMO

Vaglica F.[1], Buttacavoli M.[1], Peri E.[1], D'Amico C.[1], Marino S.[1],Geraci F.[1], Salemi G.[2], Cancemi P.[1]

[1]STEBICEF Department, Palermo, Italy, [2]Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND), Palermo, Italy

DIPARTIMENTO DI SCIENZE E TECNOLOGIE BIOLOGICHE CHIMICHE E FARMACEUTICHE (STEBICEF)

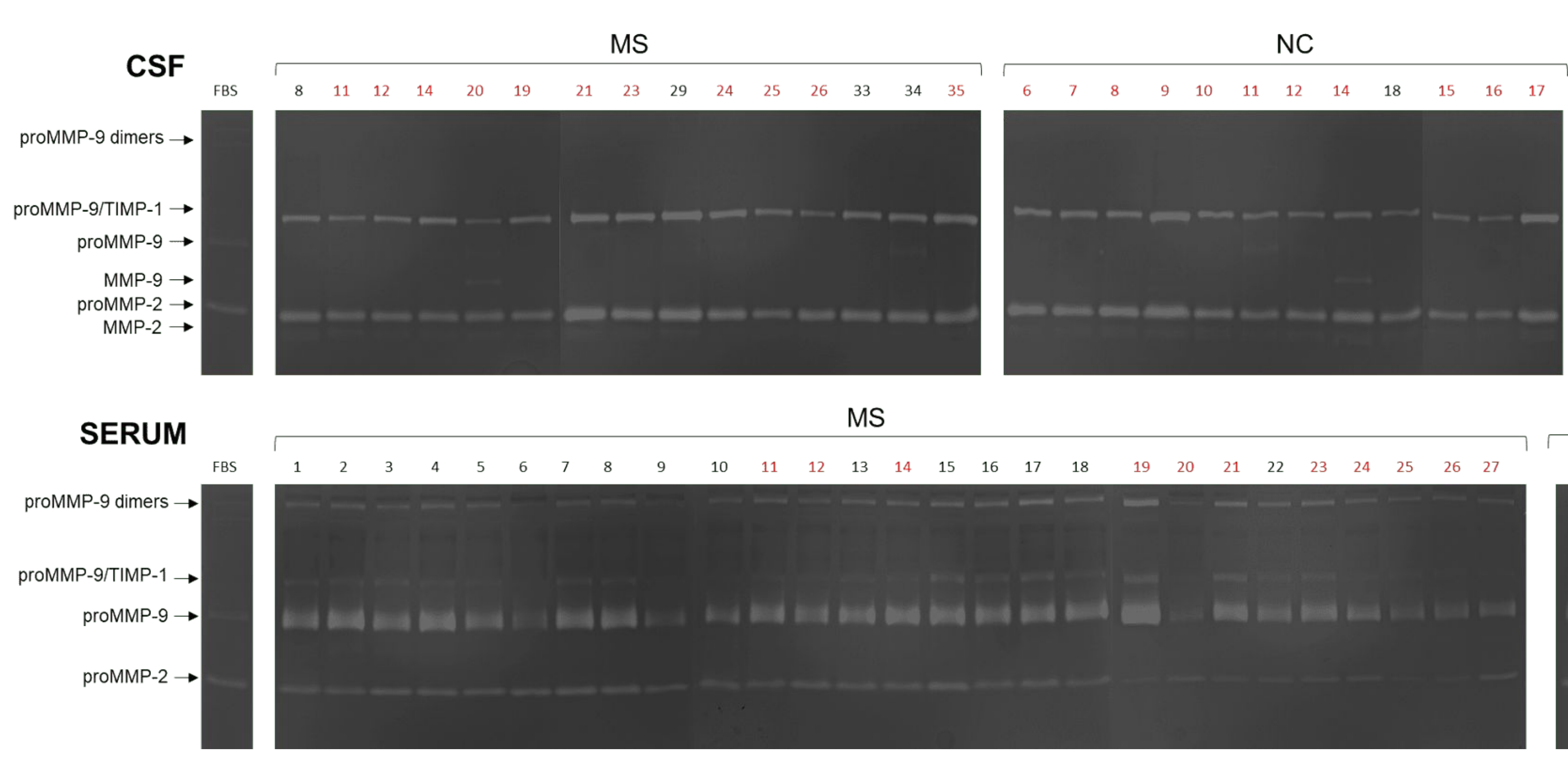
Background

In **multiple sclerosis** (**MS**), demyelination and neuroaxonal damage depend on immunocompetent cells migration into the central nervous system (CNS), due to opening of the blood-brain barrier (BBB). The BBB breakdown is mainly due to the activity of **matrix metalloproteinases** (**MMPs**), including **gelatinases MMP-2 and -9**, which have been previously proposed as candidate biomarkers for MS progression.

Aim & Methods

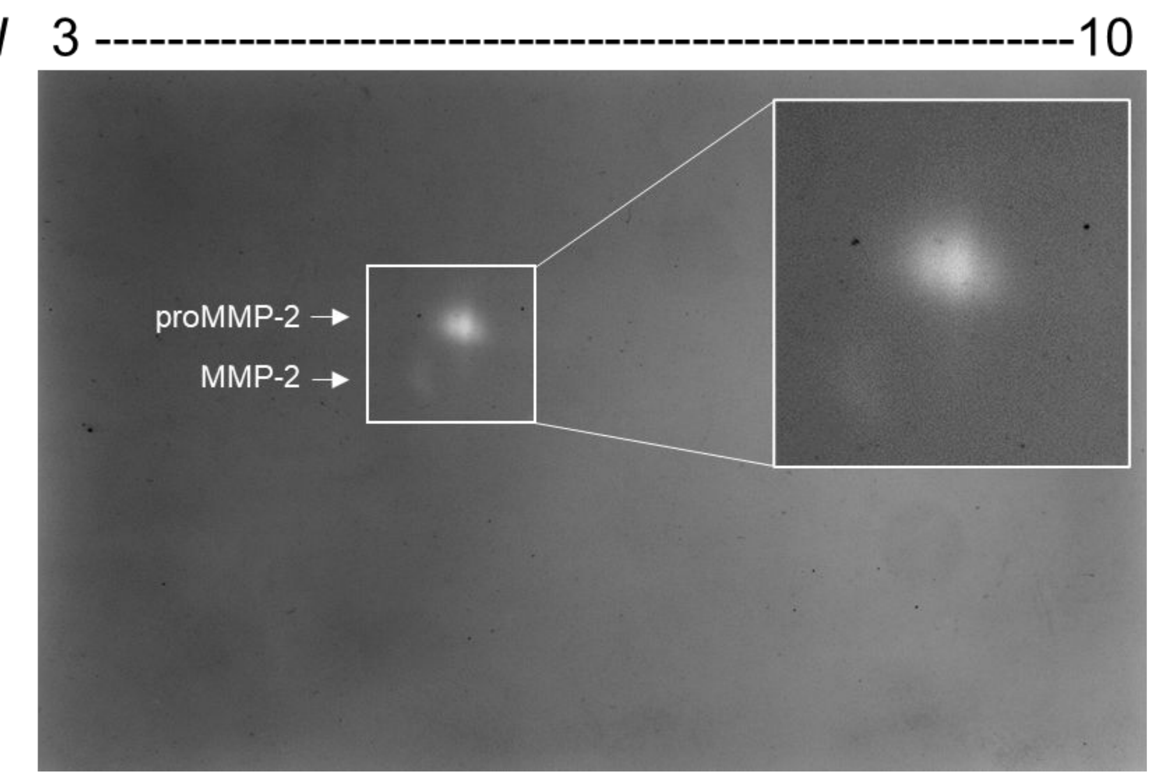
Gelatin zymography consents to detect the enzymatic activity of both pro and active forms of MMP-2 and -9 enzymes, as well as complexes of proMMP-9 with their physiological inhibitors (TIMP-1). Gelatinase activity levels have been analysed in **cerebrospinal fluid** (**CSF**) samples and in **Extracellular vesicles** (**EVs**) isolated from CSF, as well as in **sera** of patients diagnosed with **MS** or other neurological diseases, considered as controls (**NC**).

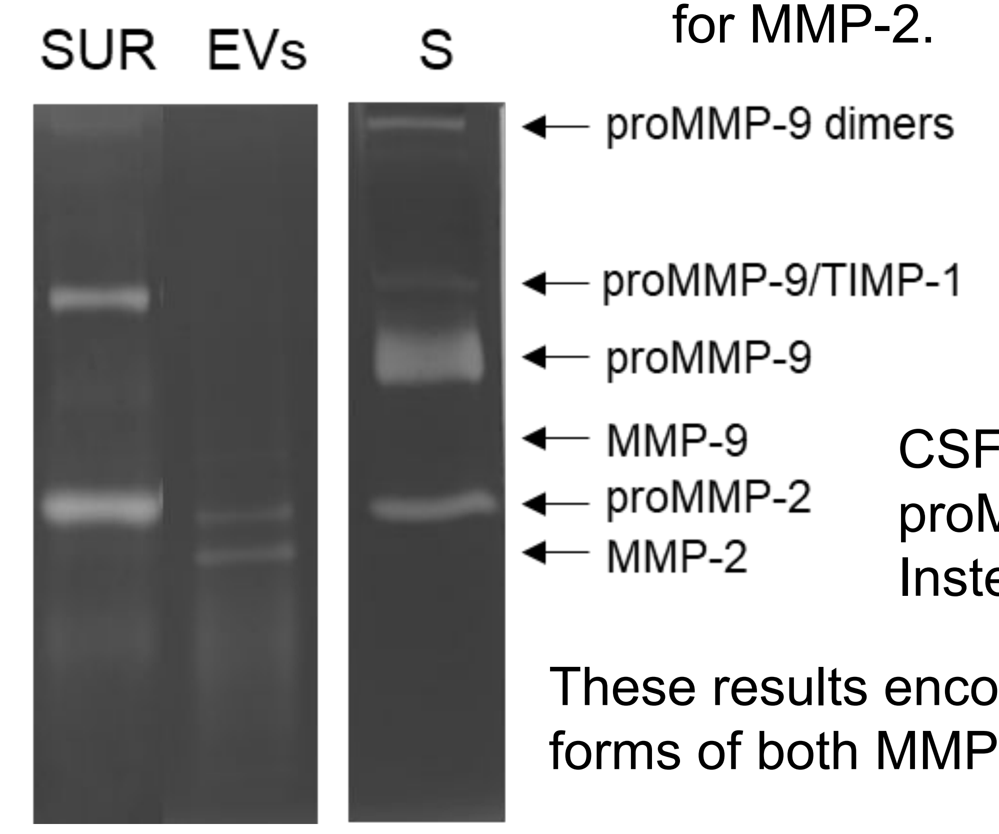
Results



Gelatin zymographies of CSF (n=16 MS, n=12 NC) and sera (n=28 MS, n=17 NC) in a cohort of 33 MS patients and 18 NC. Qualitatively, the active forms of MMP-2 and -9 were detected only in CSF samples, while the proMMP-9 dimers were revealed only in serum samples. ProMMP-9, proMMP-2 and proMMP-9/TIMP-1 were detected in both biological fluids.

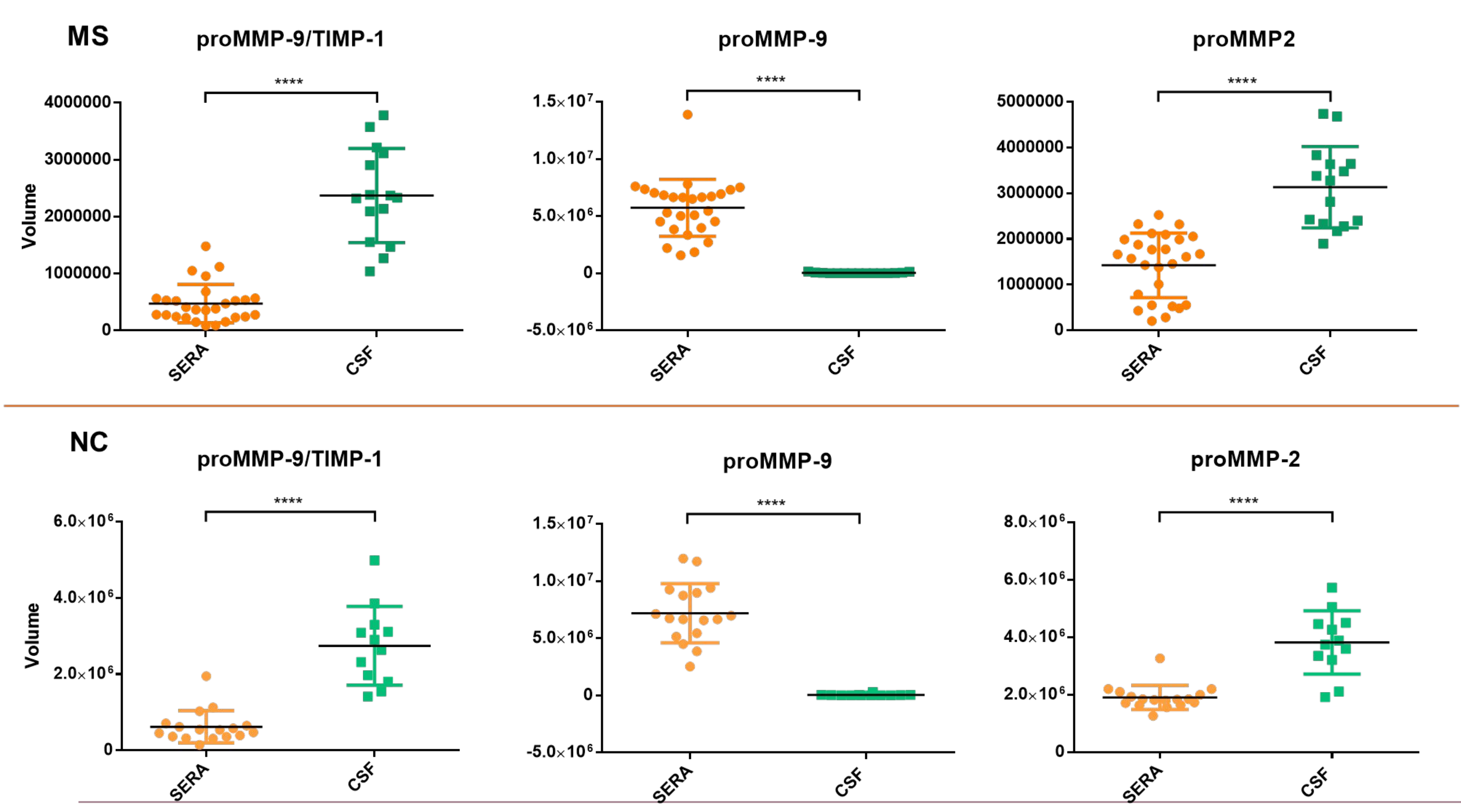
Two-dimensional (2D-IPG) gelatin zymography of a CSF samples showed three different isoforms for proMMP-2 and a spot for MMP-2.





CSF-derived **Extracellular Vesicles** (**EVs**) present both proMMP-2 and its active form, as well as the activated MMP-9. Instead, proMMP-9/TIMP-1 complex is absent.

These results encourage the hypothesis of a possible enrichment of the active forms of both MMP-2 and MMP-9 within EVs.



Scatter plots showing the **expression levels** of **MMPs** evaluated by gelatin zymography in all CSFs and sera samples (MS and NC). The quantification of lytic bands revealed that proMMP-9 levels were significantly higher in sera ($p<0.001$), while proMMP-9/TIMP-1 proMMP-2 levels were significantly higher in CSF ($p<0.001$).

Conclusion

MMP-2 and MMP-9 could be potential **biomarkers** for monitoring MS disease activity. Moreover, a shift in proMMP-9/TIMP-1 balance towards proteolytic activity of MMP-9 could be relevant in MS immune dysregulation. Further investigations are necessary to disclose the functional role of MMP-isoforms and the EVs in multiple sclerosis progression.

fabiola.vaglica@unipa.it

INTERACTION BETWEEN β-ARRESTIN1 AND M2 MUSCARINIC RECEPTORS IN GLIOBLASTOMA CANCER STEM CELLS: IMPLICATION IN CELL PROLIFERATION AND SURVIVAL

SAPIENZA UNIVERSITÀ DI ROMA

Guerriero C.[1], Scanavino G.[1], Masi I.[2], Lupo G.[1], Matejcek M.[1], Rosanò L.[2], Tata A.M.[1]

[1]Dept. of Biology and Biotechnologies Charles Darwin, University of Rome Sapienza, Rome, Italy;
[2]Institute of Pathology and Molecular Biology, CNR, Rome, Italy.

Background

Glioblastoma (GBM) is characterized by a high cellular heterogeneity and by the presence of the subpopulation of tumor stem cells (GSCs). This subpopulation represents the tumorigenic component of the tumor mass. Our previous data showed that the selective M2 muscarinic receptor activation, mediated by the orthosteric agonist APE and dualsteric agonist N-8-Iper, induces cytotoxic effects, decreasing cell proliferation and inducing cell cycle arrest in both GBM cell lines and GSCs [1-2]. A family of proteins important in regulating GPCR activity, such as M2 receptor, is the β-arrestin family. In 2020 R. J. Lefkowitz's group has demostrated the direct interaction between β-arrestin1 and M2 receptor, through cryo-electron microscopy. They have also demonstrated that the interaction of β-arrestin1 with M2 receptor is critical for desensitization and internalization of the receptor [3]. Our study may contribute to a better understanding of the mechanisms downstream of M2 muscarinic receptors and the role of β-arrestin1 in GBM cell models.

Aim & Methods

The project aims to investigate the possible interaction between β-arrestin1 and M2 muscarinic receptor in both GBM cell lines and GSCs, and the possible modulation of β-arrestin1 after M2 muscarinic receptor activation, through western blot analysis and protein localization in β-arrestin1 transfected cells. The other objective of the work is to analyze the possible role of β-arrestin1 in the kinetics of the M2 muscarinic receptor. To do this, we performed transfection with the two plasmids (M2-flag and β-arrestin1-EGFP) in order to analyze the localization of the two proteins by fluorescence microscopy in the absence and presence of M2 agonist stimuli.

Conclusions

The preliminary data here reported, show that activation of the M2 muscarinic receptor produces a general down-regulation of β-arrestin1 and its translocation from the nucleus to the cytoplasm. Moreover the analysis of cells M2-transfected, suggest a progressive expression of M2 muscarinic receptor on the plasma membrane after M2 selective stimulation. The future perspectives will be to better clarify the interaction between these two proteins and to understand the role of β-arrestin1 in the modulation of the effects downstream M2 receptor activation.

References

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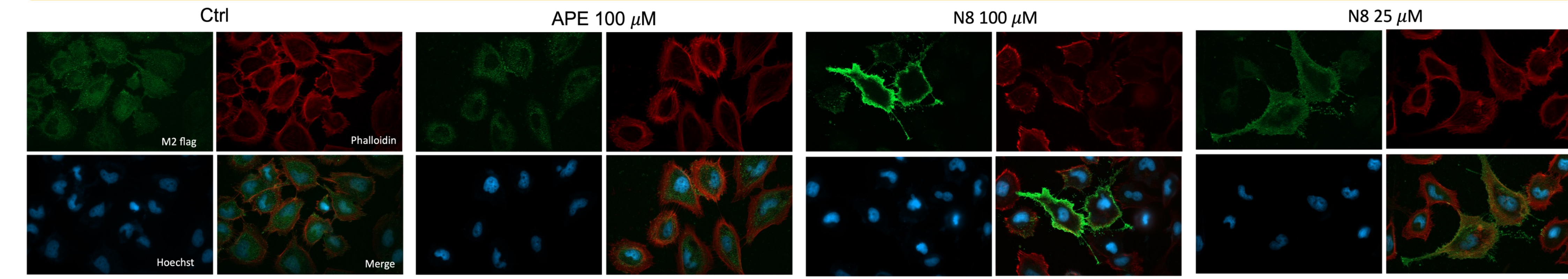
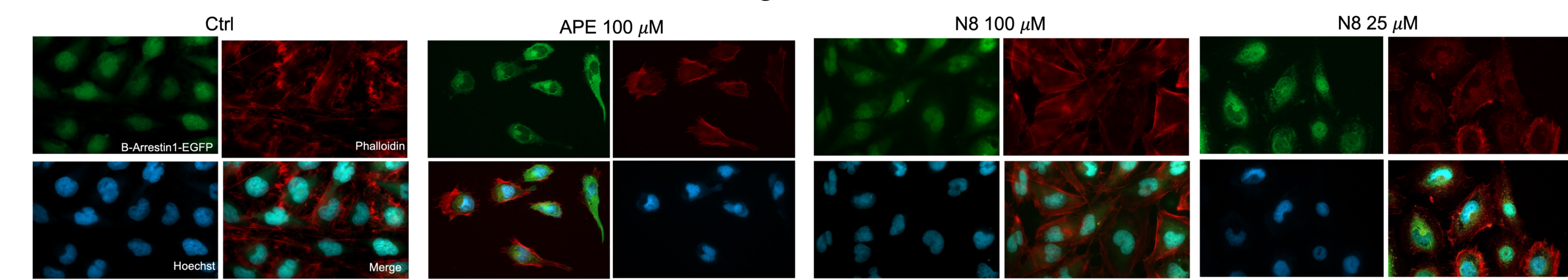
3. D. P. Staus *et al.*, «Structure of the M2 muscarinic receptor–β-arrestin complex in a lipid nanodisc», *Nature*, 2020, doi: 10.1038/s41586-020-1954-0.

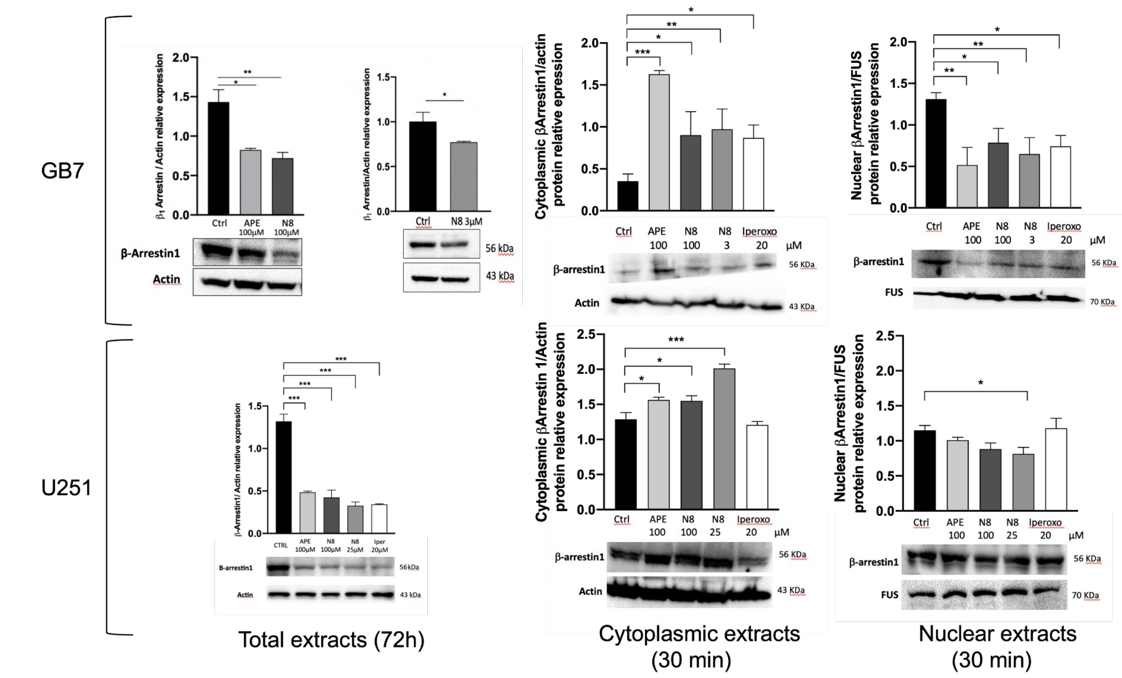
Results



The above fluorescence pictures are related to U251 cells transfected with M2 receptor-flag vector (green), treated for 30 min with 100 µM APE, 100 µM N-8-Iper (N8) or 25 µM N8. Phalloidin staining (red) was used to detect the cytoplasm and the actin located under plasma membrane; the nuclei were stained with Hoechst 33342. Analysis shows a progressive expression of M2 receptor from cytoplasm to membrane, especially in cells treated with N8 agonist.



The above fluorescence pictures are related to U251 cells transfected with β-arrestin1-EGFP expression vector (green), treated for 30 min with 100 µM APE, 100 µM N8 or 25 µM N8.-Iper. Phalloidin staining (red) was used to detect the cytoplasm, and the actin located under plasma membrane; the nuclei were stained with Hoechst 33342. Analysis shows the progressive localization of β-arrestin1 from the nucleus to the cytoplasm, after M2 agonists treatment.



Western blot analysis shows a decrease of β-arrestin1 protein expression after 72 h of treatment with high and low doses of both agonists in U251 and GB7 cell lines.
In both cell lines, after 30 min of M2 receptor activation with APE and N8, it is evident a decreased expression of β-arrestin1 localized in the nucleus and a parallel increased expression in the cytoplasm of GB7 and U251 cells,

claudia.guerriero@uniroma1.it

Aquaporin deficiency affects morphological and glycosylation patterns in the gastric cells of a murine model

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Mentino D.[1], Nicchia P.[1], Frigeri A.[2], Desantis S.[3], Guglielmi M.V.[1], Semeraro D.[1], Scillitani G.[1], Mastrodonato M.[1]

[1]University of Bari Aldo Moro, Dept. of Biosciences, Biotechnologies and Enviromental, Bari, Italy
[2]School of Medicine, Department of Translational Biomedicine and Neuroscience , University of Bari Aldo Moro, Bari, Italy
[3] Dept. of Precision and Regenerative Medicine and Ionian Area, Section of Veterinary Clinics and Animal Productions, University of Bari Aldo Moro, Bari, Italy

DIPARTIMENTO DI BIOSCIENZE, BIOTECNOLOGIE E AMBIENTE
DIPARTIMENTO DI MEDICINA E NEUROSCIENZE,
DIPARTIMENTO DI MEDICINA RIGENERATIVA

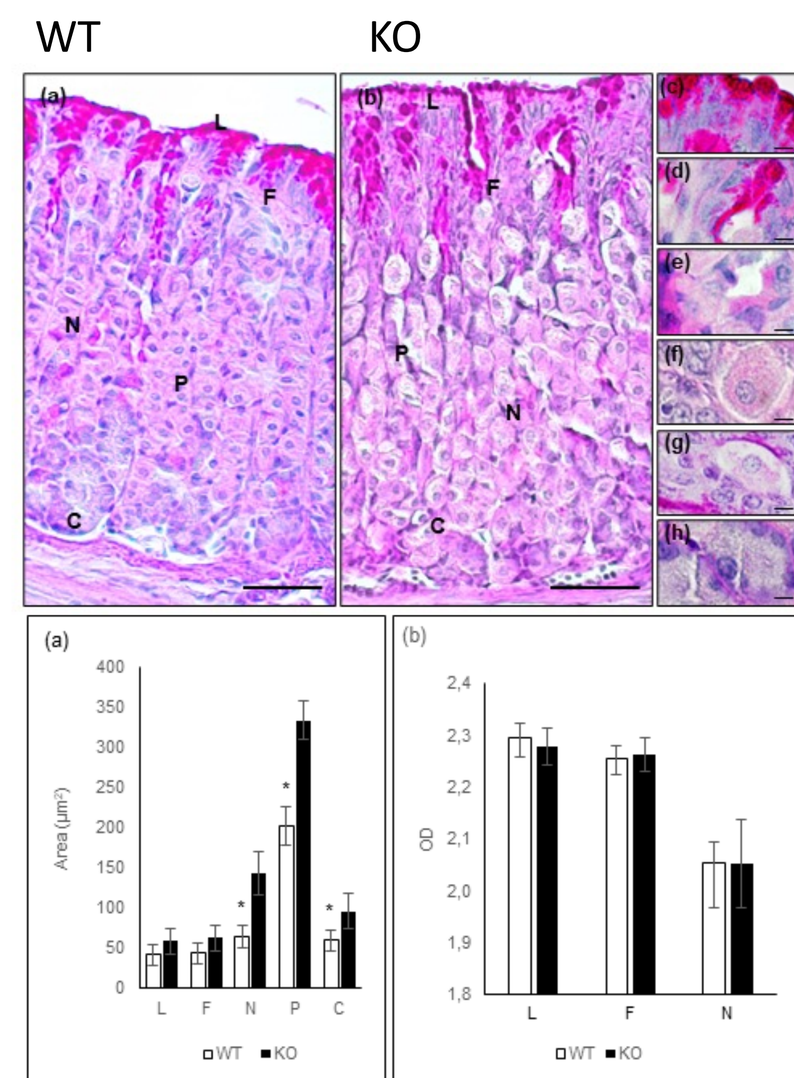
Background

Glycosylation plays a fundamental role in determining structure and its alterations of glycosylation patterns can be associated to several pathological conditions[1]. AQPs are important for water transport in the gastrointestinal tract[2] and changes in their expression could cause in disorders and be used as therapeutic targets.

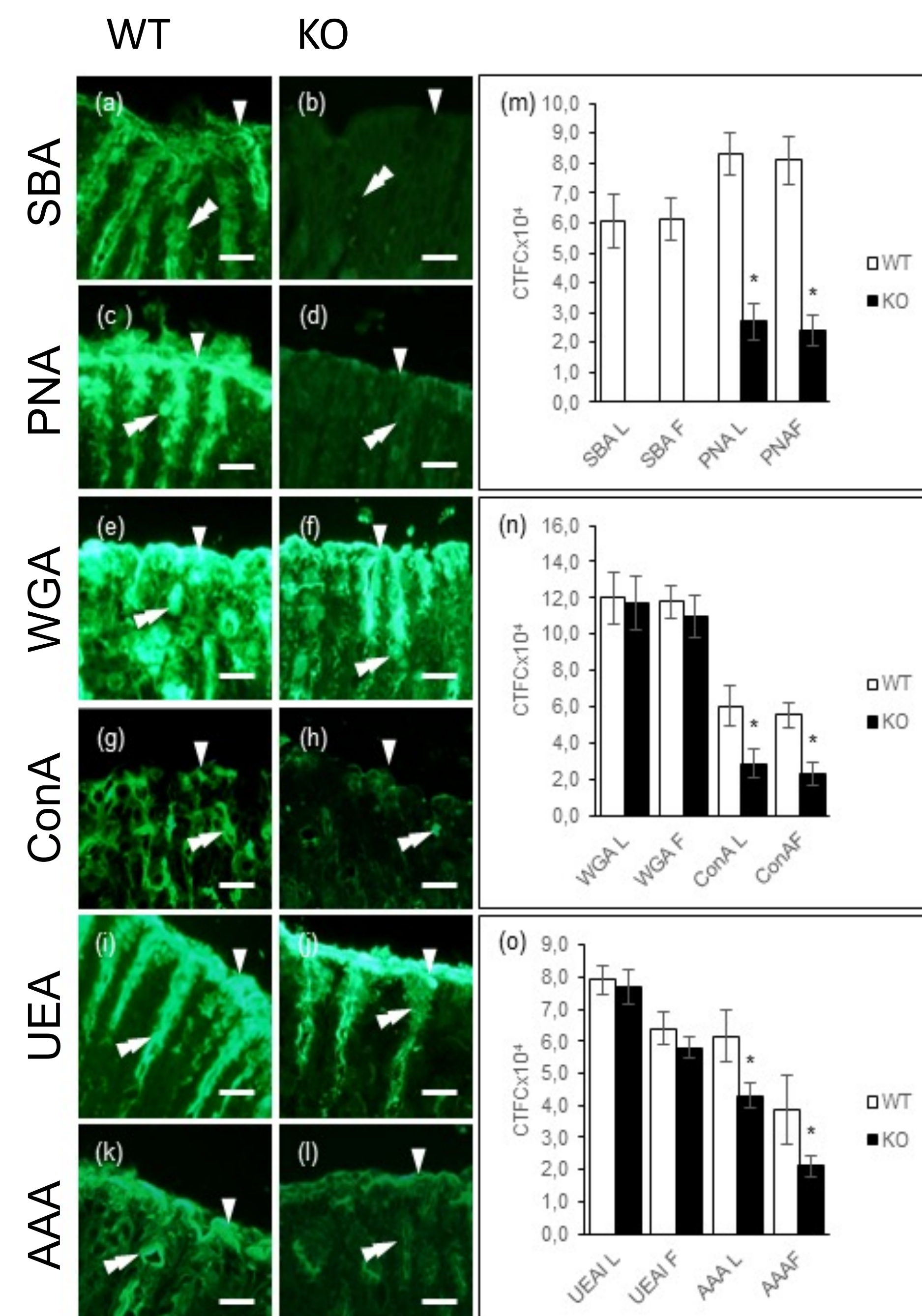
Aim & Methods

We aimed to assess whether the lack of AQP4 affects the glycosylation of secreting cells of the murine gastric glands. We compared WT and KO mice with an array of histological, histochemical and immunohistochemical techniques to evaluate in situ qualitative and/or quantitative alterations in the glycophenotypes of the mucins secreted by the mucous cells as well as those of the β-subunit of the H+/K+-ATPase in the parietal cells.

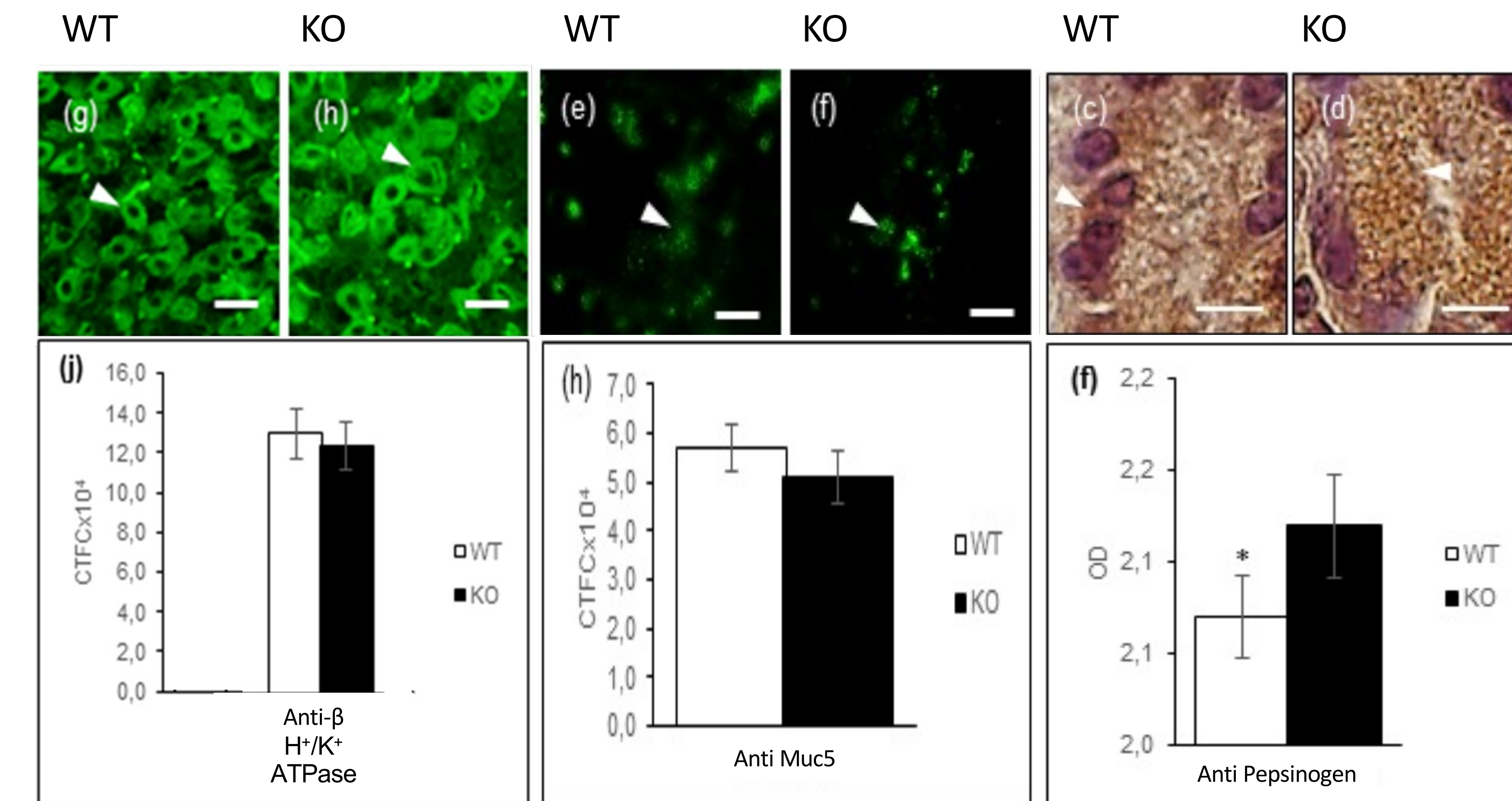
Results



PAS-HE: The parietal cells appears with faint diffuse PAS-positivity or around a ring shape nucleus.



SBA: positivity was observed only in mucous luminal (L) and foveolar (F) cells of the WT.
PNA: positivity in the mucous and neck; binding intensity significantly decreased in the KO group.
WGA: intense binding to glycosaminylated residuals was observed in mucous cells.
ConA: positivity was observed in the mucous cells only; the WT group resulted significantly higher for L and F cells.
UEA-I: was observed in L and F cells, with similar intensity between WT and KO.
AAA: stained L and F cells was stained with intensity that decrease significantly in the KO group.



Immunostaining of H+/K+-ATPase assay resulted intense in both WT and KO groups

Anti-Muc5A was observed in both WT and KO groups with similar intensity.

The pepsinogen granules appeared larger and stained significantly higher than in the WT group.

Conclusion

Differences in morphology and glycan composition of glandular cells between KO and WT mice could have a compensatory effect to allow physiological levels of secretion. This could compromise the mucosal integrity and evolve into more complex pathological conditions.

References

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donatella.mentino@uniba.it