Universidad del País Vasco
Euskal Herriko Unibertsitatea

KIMIKA FAKULTATEA
FACULTAD DE QUÍMICA

University of the Basque Country / Euskal Herriko Unibertsitatea

Faculty of Chemistry / Kimika Fakultatea

Chemistry degree

DEGREE FINAL PROJECT

Semiquantitative systematic study on differential protein expression levels of ALS patients and healthy volunteers under the effect of cellular modulators

ISS BIODONOSTIA, Mitochondrial Health and Longevity.
Group principal: Ian James Holt

Author: Mirane Florencio Zabaleta

Director: Eider San Sebastian Larzabal

2022ko uztaila, Donostia

GIPUZKOAKO CAMPUSA
CAMPUS DE GIPUZKOA
Pº Manuel de Lardizabal, 3
20018 DONOSTIA-SAN SEBASTIÁN
GIPUZKOA

LABURPENA

Alboko esklerosi amiotrofikoa (AEA) hausazko mugimendua kontrolatzen duten nerbio-zelulak kaltetzen dituen giza-gaixotasun bat da, zeinak paralisia eta heriotza suposatzen duen. Bi AEA gaixotasun ezagutzen dira esporadikoa eta familiarra edo genetikoa. Gaixotasunen kontrol eta prebentzio zentroaren arabera, oraindik ez da AEAren jatorria ezagutzen eta oraindik ez dago tratamendu posiblerik. Jakina da proteinen degradazio sistemek rol garrantzitsua betetzen dutela adinarekin zerikusia duten gaixotasun neurodegeneratiboetan neurona motoreen biziraupenarekin, hori dela eta, sistema hauekin zerikusia duten bidezidor, gene edota proteinetan jarri da atentzioa. Horretarako AEA genetikoaren kasuan bereziki, zientzialariak mutazio hauek txertatzen ari dira animalietan tratamendu desberdinak probatu ahal izateko.

Lan hau AEA esporadikoan ardazten da, zeina proteinen-formazioan ematen diren arazoetan izan dezakeen jatorria. Hori horrela, proteinaren espresioaren inguruko azterketa sistematikoa gauzatu da bai gaixo zein boluntario osasuntsuen zelulekin esperimentu desberdinak gauzatuz, bai bidezidorren moduladoreekin tratatu aurretik eta ondoren. Azterketa hau AEA gaixotasunean paper garrantzitsua izan dezaketen proteinekin gauzatu da, batez ere proteinen degradazio sisteman zentratuz. Zentzu honetan, akats mitokondrialak ere arazo larriak sor ditzakete homeostasi zelularrean, zeinak zelula barneko oreka eta funtzionamendu egokia mantentzen duen. Gaixotasun batek gorputzaren zeluletan eragiten duenean, hauek estres egoera bat jasaten dute eta zelulak egoera hau kudeatzeko gai ez badira, kontrola galtzen dute, gaixotasunaren egonkortzearekin zerikusi zuzena izan dezakeena. AEA esporadikoaren kausen artean estresaren aurreko erantzuna aurkitu dezakegu ere, proteinen degradazio prozesuak eta glukosaren metabolismoko arazoekin batera. Bidezidor guzti hauek, gehienak behintzat, erlazionatuak daude eta beraien arteko elkarlan egokia beharrezkoa da zelulak ondo funtzionatu dezan.

Hori horrela, lan honek aurretik aipatutako prozesuetan parte hartzen duten hainbat proteinen adierazpen-maila aztertzen da sistematikoki. Irakurleak ikusiko duen bezala, proteina batzuk bide zehatz baten parte bezala sailkatzen dira, baina, hauek hainbat prozesu zelularretan parte hartu dezakete aldi berean. Estres integratzailearen erantzunari dagokionez, lau proteinen espresio-maila ikertu da (BIP, DELE1, eIF2αP eta CHOP), proteinen degradazio bidezidorrei dagokionez zazpi proteina (Ub, Ub askea, UBQLN2, VCP, LC3B, P62 eta CD147) eta glukosaren metabolismoaren disfuntzioari dagokionez beste lau proteina ikertu dira (HxKI, HxKII, MTCO1 eta MTCO2).

Horretaz gain, agente kimiko batzuen (moduladore edo tratamendu bezala adieraziak) efektua proteinen espresioan ere ikertua da. Bereziki AEA gaixoen zelulak glukolisi, autofagia edota proteosoma prozesuen inhibitzaileen efektua aztertu nahi da, bide hauetako arazoek estres egoerak ekar ditzakeelako eta ondorioz zelulen funtzionamendu okerra. Tratamendu horien artean bi glukolisi inhibitzaile daude (2DG eta KA), beste konposatu bat autofagia gelditzen duena (CLQ) eta proteina mitokondrialen inhibitzaile bat (Actinonin).

Ikerketa Western Blot (WB) analisien bitartez gauzatu da, bai AEA gaixo eta boluntario osasuntsuen zelulekin. Bereziki emaitza interesgarriak lortu dira UBQLN2 eta estres integratzailearen erantzunaren presentzia AEA zeluletan.

SUMMARY

Amyotrophic lateral sclerosis (ALS) is a serious human condition that gradually attacks nerve cells that control our voluntary movement, leading to paralysis and death. Two types of ALS conditions have been described: Genetic ALS and Sporadic ALS. According to the Centers for Disease Control and Prevention (CDC), nobody knows for sure why ALS occurs, and there is currently no cure. It is well known that both in aging and neurodegenerative disorders, protein clearing systems play a critical role in motor neuron (MN) survival, and therefore current efforts are focused on identifying specific genetic and/or protein disorders in various mechanisms which clear proteins from the cell. In this sense, in the case of genetic ALS, scientists are trying to identify genetic mutations in patients, in order to transfer that gene to animals for drug treatment testing.

The present work focusses on Sporadic ALS (sALS) condition. Since a fault in protein formation could be the origin of this condition, a systematic study on the protein expression level of both patients with sporadic ALS condition and healthy volunteers is presented, both before and after the administration of the corresponding cellular pathway modulator. The systematic study of protein expression level has been focused on those proteins playing a key role in those mechanisms which clear proteins from the cell. In this sense, mitochondrial malfunction can produce severe problems in the homeostasis process, which controls the stability in the inner cellular space. When a disease is triggering the cells of our body, they are submitted to a stress situation, if the cells are incapable to handle that situation, they start to lose control which carries in this case mitochondrial malfunction, that can have a straight relation with the disease source. Within the possible causative pathways, we can find stress response, protein degradation or glucose metabolism dysfunction related processes. Most of these routes are correlated and their correct interplay is a requisite for the proper functioning of the cell.

Therefore, the present work analyzes systematically the expression level of multiple proteins involved to some extent in the processes mentioned above. As the reader will see, some proteins are classified as members of a specific pathway. However, it is important to notice that they may be playing part in more than one cellular process simultaneously. In relation to integrated stress response (ISR), the expression level of four proteins has been analyzed (BIP, DELE1, eIF2αP, CHOP), in relation to protein degradation pathways, the same has been than with seven proteins (Ub, Free Ub, UBQLN2, VCP, LC3B, P62, CD147), and, in relation to dysfunction in glucose metabolism four additional protein have been analyzed (HxKI, HxKII, MTCO1, MTCO2).

Importantly, the effect on protein expression of various chemical agents (referred as modulators or treatments) was also studied. In particular cells of ALS patients treated with inhibitors of either glycolysis, autophagy and proteosome pathways were analyzed, as the malfunction of those pathways can lead to stress situations. Those treatments consisted of two inhibitors of the glycolysis pathway (2DG and KA), one autophagy inhibitor (CLQ), one proteosome inhibitor (MG132) and mitochondrial protein synthesis inhibitor (Actinonin).

The study was performed through Western Blot (WB) analysis of samples derived from both ALS patients and healthy volunteers, and interesting differential expressions were identified, particularly on the expression of UBQLN2 and active state of ISR pathways in ALS.

ABBREVIATIONS

2DG	2-Deoxy-d-glucose
2DG-6-P	2-Deoxy-d-glucose-6-phosphate
Act	Actinonin
ALS	Amyotrophic Lateral Sclerosis
AMPK	5'-AMP-activated protein kinase
ATF4	Transcription Factor 4
ATF6	Activating Transcription Factor 6
Atg	Autophagy-related protein 2
ATP	Adenosine Triphosphate
BIP	Binding Immunoglobin Protein
BSA	Bovine Serum Albumine
CD147	Cluster of Differentiation 147
CDC	Centers for Disease Control and Prevention
CHOP	C/EBP homologous protein
CLQ	Chloroquine
CNS	Central Nervous System
DDM	n-dodecyl-D-maltoside
DELE1	DAP3-binding cell death enhancer 1
DMEM	Dulbecco's Modified Eagle Medium
DUBs	Deubiquitylating enzymes
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquiting-protein ligase
E4	Chain elongation factors
ECAR	Extracellular Acidification Rates
eIF2α	the alpha subunit of eukaryotic translation initiation factor 2
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
fALS	familial ALS
FBS	Fetal Bovine Serum
Free Ub	Free Ubiquitine
GADD34	Growth Arrest and DNA Damage-inducible protein-34
GAPDH	3-phosphate dehydrogenase
GWAS	Genome-Wide Association Studies
HRI	Heme-regulated eIF2α kinase
HRI	Health Research Institute
HxKI	Hexokinase I

HxKII	Hexokinase II
IRE1	Inositol-Requiring Enzyme 1
ISR	Integrated Stress Response
KA	Koningic Acid
LAMP2	Lysosome-associated membrane protein 2
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
LDH	Lactate dehydrogenase
LMN	Lower Motor Neurons
MNs	Motor Neurons
MTCO1	Mitochondrially Encoded Cytochrome C Oxidase I
MTCO2	Mitochondrially Encoded Cytochrome C Oxidase II
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMDA	N-methyl-D-aspartate
OCR	Oxygen Consumption Rate
OMA1	Mitochondrial Metalloendopeptidase
OXPHOS	Oxidative Phosphorylation
P/S	Penicillin Streptomycin
p58IPK	58-kDa inhibitor of protein kinase
P62	Sequestosome-1
PBS	Phosphate Buffered Saline
PDH	Pyruvate dehydrogenase
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3
PGI	Phosphoglucose Isomerase
PPP	Pentose Phosphate Pathway
RNA	Ribonucleic acid
sALS	sporadic ALS
TCA	Tricarboxylic Acid
Ub	Ubiquitine
UBQLN2	Ubiquilin-2
UMN	Upper Motor Neurons
uORFs	Upstream Open Reading Frames
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System
VCP	Valosin-Containing Protein
WB	Western Blot

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Neurodegenerative disorders and Amyotrophic lateral sclerosis

Neurodegenerative disorders are characterized by progressive loss of populations of neurons, which are selectively vulnerable because of metabolic or toxic disorders. Biologically, neurodegenerative diseases are typically defined by specific protein accumulation and anatomic vulnerability, they share many fundamental processes associated with progressive neuronal dysfunction and death, such as proteotoxic stress and its attendant abnormalities in ubiquitin-proteasomal and autophagosome/lysosomal systems, oxidative stress, programmed cell death, and neuroinflammation.[1] Between the neurodegenerative diseases can be found Amyotrophic Lateral Sclerosis (ALS), which involves the upper motor neurons (UMN) in the cortex and the lower motor neurons (LMN) in the brainstem and spinal cord,[2] leading to progressive paralysis and eventual death from respiratory failure.

In the course of the disease motor neurons (MNs) stop working and die, this precludes the correct transmission of information from neuron to muscles and leads to progressive paralysis. Two types of ALS conditions have been described, genetic or familial ALS (fALS) and sporadic ALS (sALS). With respect to the former, about 10% of the ALS cases are familial, this is, is originated from a genetic mutation. On the contrary, in the 90% of the cases diagnosed, no clear identifying cause of the disease is apparent, and has nothing to do with genetic history or any other specific factor in their lives, such as diet, way of life, etc. Regardless of the ALS type, currently, there is no cure and is known as the most common adult motor neuron disease, which has a life expectancy of about 2-4 years after disease onset.[3] As for some estimations done by *Johns Hopkings medicine* suggests that ALS is responsible for as many as five of every 100.000 deaths in people aged 20 or older. Population-based studies have defined that 2,16 per 100.000 persons are affected every year in Europe.[4] Other meta-analysis performed by INSERM (Institut national de la santé et de la recherche médicale) asses that the crude worldwide incidence is 1,75/100.00 person-year and after standardization 1,68/100.000 person-year.[5] Just in the Basque Country in 2021, 180 persons were affected of ALS as stated in *Noticias de Gipuzkoa* in the International Day of ALS. In this assignment I will focus on sporadic ALS possible pathogenic mechanisms, pathology and protein expression, as it is the most common type in the world population.

With respect to fALS, almost all cases have been inherited in an autosomal dominant manner,[6] namely, an abnormal gene presence in one of the 22 nonsex (autosomal) chromosomes that is inherited from one of the parents. In this sense, the most common mutations are the superoxide dismutase 1 (SOD1) gene, which was the first related gene and a mutation in chromosome 9.[7,8] Moreover, during the last years and with the technology advancement, up to 50 gene alterations have been reported to be potentially causative of ALS, such as TDP-43, which was identified by some *genome-wide association studies* (GWAS), or FUS. Also, two genes related with the proteins that are going to be analyzed; UBQLN2 and VCP, both related with protein degradation, but the second also with the autophagic process.[3] Even if ALS has a large genetic component, many of the gene variants remain unknown. Besides, there are so many cellular processes implicated in the disease progression, that the determination of the causative pathways remains as a challenging activity.[3] The complex nature, large genetic and phenotypic heterogeneity between patients makes difficult the study of the disease and a possible treatment.

1.2. Sporadic ALS

ALS is known as the most common neurodegenerative disease, but only a small percentage of the cases is familiar or has to do with genetic mutation. Most of the sALS cases have multifactorial etiology,[8] in other words, the disease is determined by several factors or in this case, cellular processes. Related or causative processes have been discovered by the identification of shared pathways between ALS patients,

that are RNA (Ribonucleic acid) metabolism, impaired protein homeostasis, nucleocytoplasmic transport defects, mitochondrial dysfunction and oxidative stress between others.[3,8,9] As it can be observed the research of the ALS disease requires to keep in mind all those processes, however, as each case is different to the other, is quite difficult to find common causes between patients which makes the objective even more challenging. *Table 1* lists some type of cellular pathways that are believed to be somehow altered in ALS pathogenesis and are going to be analyzed, such as integrated stress response (ISR), protein degradation and dysfunction in glucose metabolism. In this sense, each pathway is modulated by multiple proteins and the overexpression of each one of these proteins, would modulate the cellular pathway either via activation or deactivation of the cascade (*Table 2*). Just a few proteins of each pathway have been analyzed and quantified in the cell-lines, this can be seen in *Table 1*.

Table 1. *General cellular pathways and proteins related to ALS pathogenesis.*

Pathway	Integrated Stress Response		Protein degradation		Dysfunction in glucose metabolism	
	UPR	Heme Deprivation	Proteasomal	Lysosomal	Glycolysis	Oxidative phosphorylation
Proteins	BIP eIF2α P CHOP CD147 UBQLN2 VCP	DELE1 eIF2α P CHOP	Ub Free Ub VCP UBQLN2 CD147	Ub Free Ub LC3B P62 UBQLN2 VCP	HxKI HxKII	MTCO2 MTCO1

The first processes are related with the ISR, in this case just two are going to be analyzed, the Unfolded Protein Response (UPR) or the one known as endoplasmic reticulum (ER) stress and the one named the heme deprivation pathway. Those pathways are part of the integrated stress response, which is a signalling pathway present in eukaryotic cells, that is activated as a consequence of some physiological changes and different pathological conditions. Unfolded protein accumulation also activates ISR, as happens in UPR.[10] The second group are related with the protein degradation pathways, two have been identified and are known as proteasomal and lysosomal degradation. In the first mentioned, proteins are degraded via ubiquitin-proteasome system (UPS), and in the second, we have three distinct lysosomal pathways; Endosome recycling, phagocytic pathway and the autophagy-lysosome pathway, the one is going to be analysed extendedly[11] Finally, glucose metabolism dysfunction is going to be analysed, as sALS is thought to be related with possible defects in the central nervous system (CNS) glucose transport and metabolism, which is related with an increase of the oxidative stress and cellular homeostasis umbalance.[12] Here can be found the glycolysis process, the tricarboxylic acid (TCA) cycle the oxidative phosphorylation (OXPHOS), which derive in mitochondrial dysfunctions that are common in neuronal dysfunctions, related with the ATP formation in the TCA cycle and OXPHOS processes.[13–15]

As mentioned before in the ISR signalling many different pathways can be found (*Figure 1*), with the objective of restoring cellular homeostasis by different mechanisms. The first pathway that is going to be analysed is the UPR process, which is located in the ER and activated under a stress situation. UPR has been identified as a relevant cause of neurodegenerative diseases, as the loss of unfolded protein homeostasis entails neuronal death.[16] Moreover, pharmacological studies have stablished that the inhibition of PERK (Eukaryotic translation initiation factor 2-alpha kinase 3), the first protein in one of the UPR cascade, does aggravate the cell-death rate,[17] even if it is not totally clear in other more recent studies.[18] Because of that, this pathway must be analyzed as can have an important role in the disease onset and progress. Lately, some studies have reported that other stress pathway can be responsive of that neuronal death, which is the heme deprivation pathway. This is activated as a consequence of mitochondrial dysfunction which is related with energetic alterations in the cell that have been seen in ALS.[19] This opens other research line that must be taken into account, as it is understood as an alternative pathway, heme depletion, that could be responsible for the disease development if the UPR process is discarded.

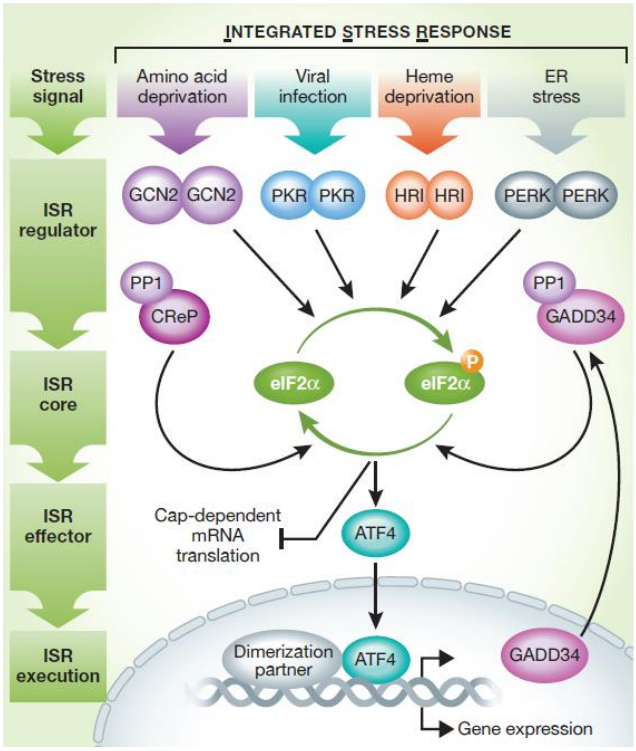


Figure 1. *General scheme of the ISR pathway.*[10]

All the stress stimuli that activate ISR have a common point of convergence, which is the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) on Serine 51.[20] The translation initiation factor mentioned before is activated just by stress stimuli and as a consequence, a reduction in global protein synthesis happens while the activation of the transcription factor 4 (ATF4) is allowed, so as to promote cell survival.[21] Nevertheless, if the cellular stress is permanent or very intense, the adaptive response capacity collapses and additional processes are activated to execute cell death. If the stress is controlled, eIF2α will be dephosphorylated and normal protein synthesis will be recovered. [22]

The ER is an important organelle that controls the protein production in the cell, mostly the secreted and transmembrane proteins.[22,23] The number of unfolded and misfolded proteins is decisive for the ER homeostasis and is controlled by the protein translation, folding and degradation processes. The ER stress is created by the accumulation of excessive unfolded or misfolded proteins and can be observed either in normal physiological conditions nor disease conditions, where gene mutations or disturbances in metabolic and redox status can create the protein excess.[22,24] So as to control the unfolded proteins, there is a kind of quality control that ensures that protein folding has been done correctly and then, protein leave the ER to go to the Golgi apparatus.[23] If not, unproperly folded proteins are retained in the ER and are taken to other pathways like endoplasmic reticulum associated degradation (ERAD) that is based on the UPS or autophagy.

The inability to handle the folding of the proteins that enter the ER produces a stress situation (ER stress) that is very common in the neurodegenerative diseases[25] and the one is mastered by the activation of rapid-response mechanisms, as the UPR. This activation causes a shutdown of the global protein synthesis and unfolded protein control mechanisms are activated, by the degradation of those proteins increasing the production of ER chaperones, like GRP78 or BIP (binding immunoglobin protein),[23] the first identifies terminally misfolded proteins and takes them to the refolding process, whereas the second activates PERK if the refolding process is not sufficient. If the stress is prolonged, apoptosis can be induced.[26] The UPR improves protein folding by up-regulation of ER chaperones and provides unfolded protein degradation by promoting ERAD and reducing the number of new proteins entering the ER by inhibiting protein translation.

Like that ER stress is eliminated, which is critical for the cell to function and survive.[23] The UPR can be seen as a signal transduction pathway that is based on two components: First, the stress sensors in the ER membrane (PERK, IRE1 and ATF6) and then, downstream transcription factors like eIF2α that reprogramme gene expression toward stress maintenance or apoptosis induction.[27]

There are three sensors in the ER lumen that manage UPR and the stress situation. As seen in *Figure 1*, this are inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6). We are going to analyze just the cascade started by PERK, as is the one that has been directly related with ALS.[25] This is a type I transmembrane protein situated in the ER, that identifies the unfolded proteins accumulated in the ER lumen. In the absence of ER stress, GRP78 binds to the luminal domain of PERK and prevents the activation. Upon ER stress BIP (GRP78) dissociates and PERK is activated through oligomerization and trans-phosphorylation.[28,29] The sensor phosphorylates and inactivates the α subunit of eIF2, followed by the inhibition of the protein synthesis. Moreover, the phosphorylation of PERK is an equilibrium controlled by its regulatory subunit GADD34 (growth arrest and DNA damage-inducible protein-34) and by its endogenous inhibitor, p58IPK. [23,30–32] Apart from that, the phosphorylation of eIF2α enhances ATF4, which induces its target genes like C/EBP homologous protein (CHOP), which is a transcription factor involved in the induction of apoptosis, between others.[33]

Simply said and as can be seen in *Figure 2*, PERK modulated pathway is based on first the identification of misfolded/unfolded protein accumulation in the ER lumen, then ER chaperone BIP will be formed and then these will attach to PERK by oligomerization and transphosphorylation. Then the cascade is going to be activated and eIF2α will be phosphorylated by GADD34, which leads to an inhibition of the protein translation. Some mRNAs are encoded by ATF4, which encode small upstream open reading frames (uORFs) in their 5'-UTR and like that, ATF4 activates the expression of CHOP, GADD34 and additional factors that are important for amino acid metabolism and redox control.[34]

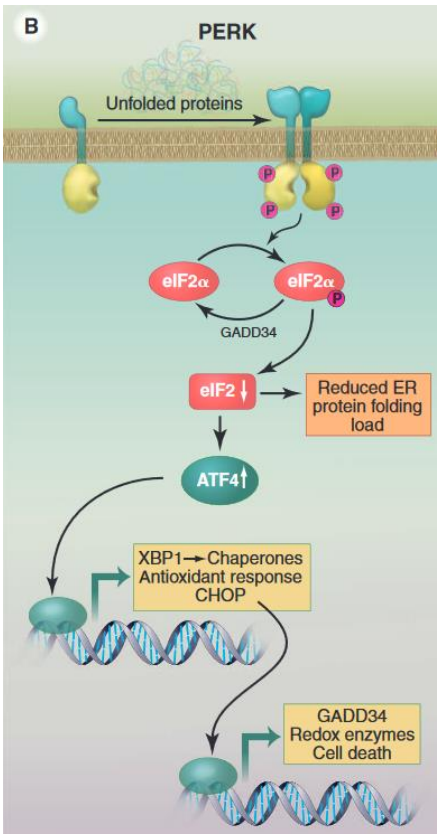


Figure 2. PERK cascade.[35]

Once analyzed the pathway and the proteins related, the expression of those must be taken into account as can give us important information about the pathway and about the situation in the general cell functioning. For that, the meaning of the expression has been described in the table below (see *Table 2*) and after are going to be discussed in order to the results obtained.

Table 2. *Protein expression meaning for the UPR pathway.*

UPR	Protein expression meaning
BIP	BIP overexpression means that UPR pathway is active.
eIF2α P	If BIP and EIF2α P are overexpressed, UPR is active.
CHOP	If BIP, EIF2α P and CHOP are overexpressed, UPR is active.
CD147	If CD147 is overexpressed ERAD (UPR) is active.
UBQLN2	If is overexpressed, unfolded proteins are been degraded by the proteosome.
VCP	If it is overexpressed, would bind misfolded proteins form the ER and with take them to ze cytoplasm, there will be degraded by the proteosome.

Regarding Heme depletion, different proteins have been reported to be part of this pathway, but the proteins related in the pathway mentioned before are also affected by the ISR pathway, this is, when one of both processes is activated, the translation of the same proteins is induced; eIF2α is phosphorylated, and CHOP and ATF4. In this case, mitochondrial stress stimulates OMA1 (Mitochondrial Metalloendopeptidase), that cuts DELE1 (DAP3-binding cell death enhancer 1), which is accumulated in the cytosol and interacts with HRI (Heme-regulated eIF2α kinase). This causes the phosphorylation of eIF2α and the same process mentioned before happens.[19] Anyway, in the case of this pathway the next protein cascade is activated, the mitochondrial protease OMA1, DELE1, HRI, eIF2α and CHOP respectively. This cascade has been reported in the last years, by the silencing of HRI and DELE1 experiments.[36] Because of that is important to take it into account as an alternative route to PERK, as it has been seen that has a direct effect on CHOP expression. However, there is little information about that cascade and specially about the protein DELE1, but this is the missing pathway that is triggered by mitochondrial stress, that has nothing to do with the UPR.

The mechanic features of the cascade (see *Figure 3*) are first the stress-induced activation of OMA1, which causes DELE1 to be cleaved, that happens in histidine 142,[19] into a short form ($DELE1_S$) that is accumulated in the cytosol, even if has also been observed that non stressed cells can also create $DELE1_S$. Then, this protein binds to HRI and activates it via its C-terminal portion. Finally, by its TRP (Tetratricopeptide repeat) domains HRI interact with eIF2α inducing its phosphorylation and consequently CHOP translation.[36] This process sets off the ISR and this can be beneficial or not depending on the mitochondrial stress. Apart from the function just mentioned, DELE1 can also alert the cell about perturbations that affect to the outer mitochondrial membrane, specifically in the pores, by interacting with HRI in its mature form, this is, $DELE1_L$.[37]

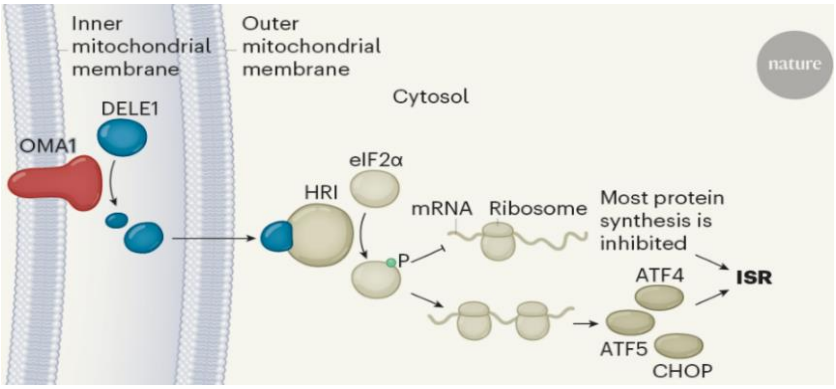


Figure 3. *ISR Pathway.*[38]

At first, HRI was thought to be activated by heme deprivation, where HRI is activated by its own autophosphorylation.[39] So, it seems that HRI can have different roles, as it can also act as a heme sensor, in which case heme acts directly with HRI and inactivates it.[40] However, as mentioned above, it can also be activated by heme-independent mechanisms, as it is going to be analyzed during the work. In various researches, has been reported that HRI knock out increases ER stress,[41] But specifically in neuronal cells, HRI has been found to mediate the translation of GluN2B, a subunit for the N-methyl-D-aspartate (NMDA) which is an important receptor for neuronal activity.[42] Apart from that, HRI is activated distinctly to other kinases as its direct activators provide targeting a specific kinase upstream of eIF2α phosphorylation without globally affecting the downstream activity of the other kinases, this is, without activating UPR.[1]

Once analyzed the pathway and the proteins related, the expression of those must be taken into account as it can give us important information about the pathway and the general situation of the cell functioning. For that, the meaning of the expression has been described in the table below (see *Table 3)* and after, is going to be discussed depending on the results.

Table 3. *Expression meaning for ISR.*

ISR	Protein expression meaning
DELE1	When the pathway is activated, DELE1 is cut. If DELE1 is overexpressed, ISR is active. DELE1$_S$: If is overexpressed, ISR is active.
eIF2α	If is underexpressed, ISR is active.
eIF2α P	If is overexpressed, ISR is active.
CHOP	If DELE1, eIF2α P and CHOP are overexpressed, ISR is active.

The two major degradative systems in eukaryotic cells are the proteasome and the lysosome. The first is based in UPS as this is responsible for degrading intracellular proteins, or even transmembrane proteins if they are extracted from the membrane into the cytosol. The lysosome degrades membrane and endocytosed proteins, even digests cytosolic proteins through autophagy.

In neurons as well as other eukaryotic cells, intracellular proteins are primarily degraded by UPS, and membrane proteins by the lysosome system. Ubiquitylation tags proteins for proteasomal degradation and vesicular trafficking. Several classes of UPS factors are involved in presenting substrates to the proteasome: the ubiquitylating enzymes (E1–E4), deubiquitylating enzymes (DUBs), shuttling factors and chaperones. The action of these factors can differ for each substrate, and there will be a single potential configuration.[43] A simple scheme is described in the following lines (See *Figure 4*).

Ubiquitylation is a post-translational modification, by the formation of an isopeptide bond between the C-terminal of the ubiquitin and a lysin residue. The ubiquitylation system is formed by four different enzymes: E1–E4. The process is based on first, binding covalently ubiquitin to E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction. Then, it is transferred to the E2 (ubiquitin-conjugating enzyme). After that, E3 enzyme (ubiquitin–protein ligase) transfers the ubiquitin from E2 to the substrate protein. Once monoubiquitylation happens, E3 can elongate the ubiquitin chain by creating isopeptide bonds between ubiquitin proteins. The E4 enzymes (chain elongation factors) are a subclass of E3-like enzymes that only catalyse chain extension, as polyubiquitylated chains are almost essential for protein degradation process. Ubiquitin has seven lysine residues, between them K48 and K63, that are available and used in vivo for chain extension. The sense of complex ubiquitylation patterns is not totally understood yet, but has been reported that K48 chains are most abundant residues and lead to degradation of the substrate by the 26S proteasome. However, monoubiquitylation and K63 chains do not specify degradation, but they have other biological functions, like marking proteins for endocytosis. The E3 is largely responsible for target recognition in this system, through physical interactions with the substrate. The large number of E3 genes in eukaryotic genomes reflects the highly specific nature of substrate recognition in UPS-mediated degradation. As

mentioned before, many ubiquitylation patterns can be described for each substrate and in *Figure 4* is shown the action of E3, E4 and DUBs. When the lysin residue is ubiquitylated (K48-polyubiquitylated), there are different ways to reach the proteasome: Generally, the protein can reach by diffusion or with the assistance of chaperones and shuttling factors, but in some cases, the proteasome can also move towards the substrate. After substrate–proteasome association, DUB and ATP-dependent unfoldase activities help the substrate to enter the proteolytic lumen of the proteasome, where the substrate is degraded.[43]

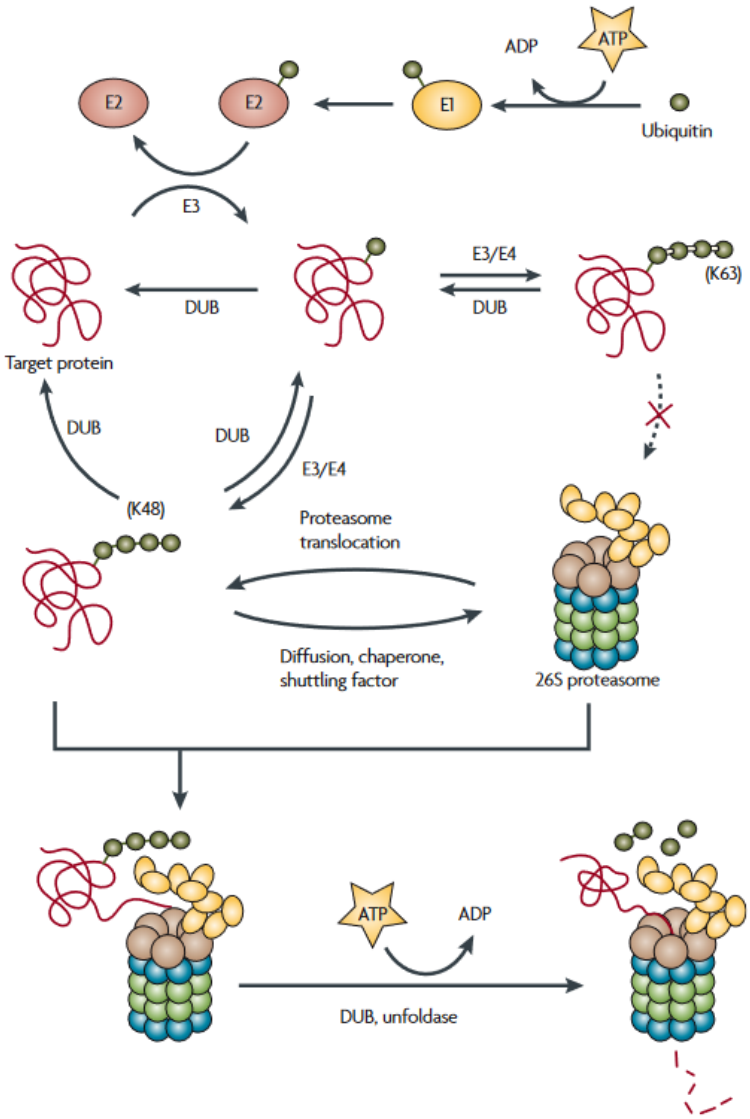


Figure 4. Proteosome degradation mechanism pattern.[43]

Some studies reported that ALS patients present Ub in intracellular inclusions as an immune-reactivity. These inclusions also obtain components of the proteosome and a disrupted proteasomal function, as has been reported in MNs, as with the disease progression there is a decrease in the proteosome subunits. ERAD degradation pathway is also linked to UPS and is been also related with ALS as mentioned before, which is activated to deal with ER misfolded/unfolded protein accumulation.[44]

Once analyzed the pathway and the proteins related, the expression of those must be taken into account as can give us important information about the pathway and about the situation in the general cell functioning.

For that, the meaning of the expression has been described in the table below (see *Table 4*) and after, will be discussed depending on the results obtained.

Table 4. *Protein expression meaning for the proteosome.*

Proteasome	Protein expression meaning
Ub	If Ub is overexpressed, more protein needs to be degraded.
Free Ub	If free Ub is overexpressed, more proteins have been degraded or there's a need for degradation.
VCP	If is overexpressed, proteosome will be active or there will be a need for protein degradation.
UBQLN2	If is overexpressed, there is a need of protein degradation, proteosome will be active.
CD147	If is overexpressed, proteins form ERAD must be degraded.

Regarding autophagy, this is a self-digesting mechanism that degrades from long-lived proteins to damaged organelles. Has been demonstrated that this process objective is to regulate different cellular functions, between them, cellular growth, differentiation, response to nutrient deficit and oxidative stress, cell death and macromolecule and organelle turnover.[45] Autophagy is a lysosomal degradation pathway that can be differed in three different processes: Micro-autophagy, Macro-autophagy and Chaperone-mediated autophagy. First, by this three mechanisms proteins or molecule are trapped by the autophagosome and then, generally, the autophagosome fuses with lysosomes, where biomolecules are degraded. In the first mechanism, a small part of the cytoplasm is internalized through lysosomal invagination. In the second, cytosolic material or even organelles are surrounded by a double-membrane structure that fuses with lysosomes, and in the last, proteins are unfolded by the chaperone translocate into de lysosome by interacting with LAMP2 (Lysosome-associated membrane protein 2).[43]

As to the mechanism, a double-membrane vesicle must be formed, which encapsulated all biomolecules or proteins for degradation. The autophagosome is formed between the ER and the mitochondria, the ones play as contact-sites[46] and the formation process needs of 16 autophagy-related proteins (Atg) and two ubiquitin-like conjugation systems that produce modified complexes that determine the autophagosome formation and size. So, to form the autophagosome, a multistep process is carried out sequentially: initiation, nucleation, elongation, maturation and thereafter fusion with the lysosome. The process starts with the inhibition of mTOR (mammalian target of rapamycin), by its phosphorylation carried out by AMPK (5'-AMP-activated protein kinase), as mTOR is a major negative regulator of autophagy. Like that a pre-initiation complex is formed, which causes the translocation to the membrane and triggers the initiation step for the assembly of the autophagosome. After that, Atg9, positive vesicles on the ER, contribute to the nucleation process by interacting with the complex mentioned before and like that, autophagosome elongation is promoted. Then, some conjugation steps happen were LC3 is attached to phosphatidylethanolamine so that the maturation of the autophagosome is provided by membrane bound LC3. Next, adaptor protein p62 (also known as: SQSTM1, sequestosome-1) between others, facilitate the degradation of misfolded and ubiquitinated substrates by binding to the membrane complex formed by LC3 and Atg-8 protein, and the autophagosome is closed, by the dissociation of an Atg proteins complex and the lipidation of LC3 (LC3B; microtubule-associated proteins 1A/1B light chain 3B) in the autophagosome. Which makes LC3B and p62 accepted markers to measure the autophagic flux. [46] Finally, autophagosome and lysosome are fused and molecules degraded (see *Figure 5*).

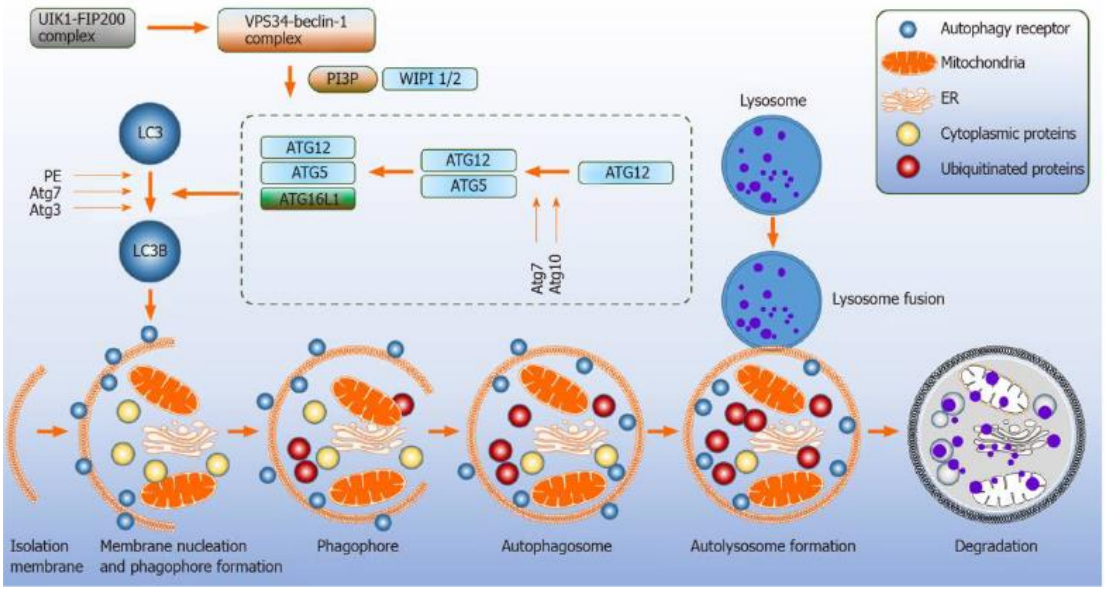


Figure 5. Scheme of the autophagy process.

The autophagy process is directly related with apoptosis or type 1 programmed cell-death and they function together in order to induce it, as it has a cytoprotective mechanism. Autophagy can promote cell survival by inhibiting the process or can also induce cell death by activating apoptosis.[47]

Once analyzed the pathway and the proteins related, the expression of those must be taken into account as can give us important information about the pathway and the general cell functioning. For that, the meaning of the expression has been described in the table below (see *Table 5)* and after the results will be discussed.

Table 5. *Protein expression meaning for autophagy.*

Autophagy	Protein expression meaning
Ub	If Ub is overexpressed, more protein needs to be degraded.
Free Ub	If free Ub is overexpressed, more proteins have been degraded or there's a need for degradation.
LC3B	If is overexpressed, means that proteins need to be degraded or that autophagosome if formed and proteins are degraded.
P62 P	If is overexpressed means that autophagy is active.
UBQLN2	If is overexpressed proteins, autophagosome is formed, proteins are degraded.
VCP	If is overexpressed, autophagosomes will be formed and proteins will be degraded.

In the CNS most of the energy obtained by glucose metabolism is used for the maintenance of action potentials and postsynaptic signalling, and specially neurons need a large amount of energy that requests an almost continuous glucose availability. This energy demand is obtained mostly via OXPHOS and glucose is the main substrate. This substrate is also important to control oxidative stress, as it is metabolized by the pentose phosphate pathway (PPP) to produce nicotinamide adenine dinucleotide phosphate (NADPH), so as to keep glutathione in an antioxidant reduced state. Energy needed for cellular basic physiological functions is produced by glycolysis and oxidative mechanism via the TCA cycle and the electron transport chain.[48]

As respect to the glycolysis pathway, is based on generating ATP (adenosine triphosphate) and NADH (nicotinamide adenine dinucleotide) by the conversion of glucose into pyruvate and the process is determined by the oxygen availability i.e., aerobic and anaerobic glycolysis processes can happen. As for

anaerobic conditions pyruvate is converted into lactate by LDH-A (Lactate dehydrogenase A) in the cytoplasm and then, this is converted to pyruvate by LDH-B (Lactate dehydrogenase B). Lactate can derive to lactic acid, and the acid generated by anaerobic glycolysis is measured by ECAR (Extracellular acidification rates). On the other side, we have aerobic glycolysis. In this case pyruvate enters the citric acid cycle via PDH (pyruvate dehydrogenase) complex, and is catabolized by OXPHOS, producing electrons that pass through the electron transport chain, to impulse protons trough the inner mitochondrial membrane. Then, an electrochemical gradient is generated, as protons are accumulated in the mitochondrial intermembrane space, enabling the production of ATP by the ATP synthase (complex V). So as to measure the oxygen utilization rate, OCR (oxygen consumption rates) is measured and mitochondrial function of the cell can be described. The energy obtained in an aerobic situation is much higher, as the conversion of glucose into lactate generates 2 ATP per glucose molecule as compared to 36 ATP per glucose molecule when the oxidative phosphorylation is used.[49]

As the process in its totality is quite complex, just a general idea is going to be explained (see *Figure 6*). Briefly, glucose is transformed by hexokinase to glucose-6-phosphate. Then, glucose-6-phosphate continues the glycolytic pathway or enters to the PPP, the one has two phases: Oxidative, that converts glucose-6-phosphate into ribulose-5-phosphate, CO_2 and NADPH, and a non-oxidative phase, where is transformed to fructose-6-phosphate between others. Instead, glucose-6-phosphate can also be converted to glucose-1-phosphate so as to produce glycogen, which is an energy reserve mainly stored in astrocytes. In the glycolysis process, glucose-6-phosphate forms fructose-6-phosphate and then, fructose-1,6-bisphosphate. After, this is converted to pyruvate and many other biomolecules by several enzymatic reactions that produce NADH and ATP. Succeeding glycolysis, pyruvate can be converted into lactate by LDH (lactate dehydrogenase) or by alanine aminotransferase to alanine. Anyway, generally this enters to the mitochondria and is converted to acetyl-CoA by PDH and simultaneously enters the TCA cycle, where acetyl-CoA is oxidated by different reactions in which reducing equivalents are form, those transfer electrons to the oxygen by the enzyme complexes of the electron transport chain, so as to produce ATP by ATP synthase.[48]

Abnormalities in glucose metabolism can affect the execution of basic cellular functions, as it can results in a reduced ATP generation. Moreover, a lower production of ATP can induce other energy generating pathways, that may increase reactive oxygen species (ROS) and oxidative stress, that can lead to neurodegeneration. In literature energy homeostasis has been reported to be disturbed as a consequence of hypermetabolism in ALS cells.[50]

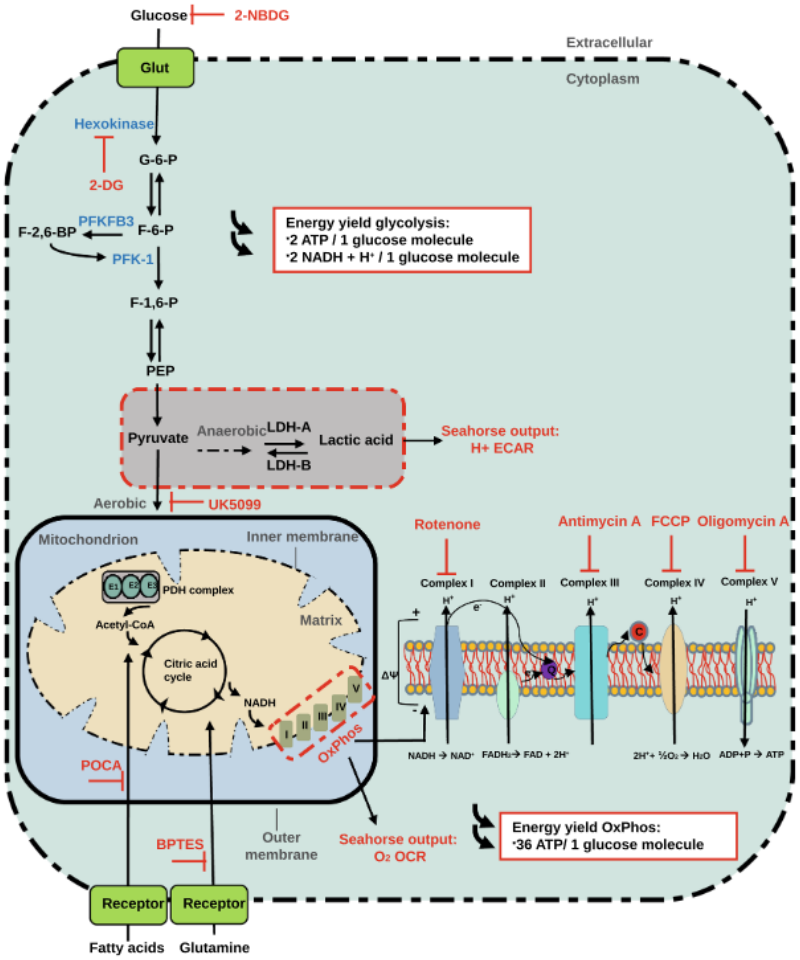


Figure 6. Glycolysis, TCA cycle and OXPHOS process.[49]

Once analyzed the pathway and the proteins related, the differential expression must be taken into account as can give us important information about the pathway and the cellular functioning. For that, the meaning of the expression has been described in the table below (see Table 6) and after, depending on the results obtained, the discussion is going to be carried out.

Table 6. *Expression meaning for Glycolysis* and Oxidative phosphorylation processes.

Glycolysis	Protein expression meaning
HxKI	If is overexpressed means that the glycolysis is activated from the first step.
HxKII	If is overexpressed means that the glycolysis is activated from the first step.
OXPHOS	**Protein expression meaning**
MTCO2	If is overexpressed, means that the fourth complex of the TCA cycle is active.
MTCO1	If is overexpressed, means that the third complex of the TCA cycle is active.

As for the literature, there are some facts that have been determining for the vulnerability of ALS cells. Firstly, its large size, with a highly developed cytoskeleton, which implies a high metabolic activity to maintain cellular functions, this should suppose a more active glycolysis or energetic activity in patient cells, which is related to high mitochondrial requirements. Secondly, is highly sensible to excitotoxic agents, as well as to alterations in intracellular calcium regulation, which has to do with ER stress as affects directly to the intracellular calcium level and neuron sinapses.[51] Thirdly, a reduced capacity for chaperone-dependent processes and function of the UPS, or protein degradation. However, even if all those facts have been observed, the underlying pathogenic mechanisms in ALS are multiple and have not been fully described.

Alterations have been identified at the level of RNA processing resulting in aberrant RNAs or toxic RNAs. High levels of oxidative stress as well as difficulties in dealing with free radicals has also been linked to the disease. Furthermore, alterations in protein metabolism with inhibition/malfunction of UPS and hyperactivation of autophagy occur. So, there should be lower proteasomal activity and higher autophagy. Disorders in proteins involved in axonal transport have also been related to ALS. Finally, alterations at the glial cell level have been reported to have an impact on MNs leading to MN degeneration.[8]

All experiments have been carried out in fibroblast, skin cells. Even if some researchers and doctors state that they are very different to neurons. However, genes that are related to diseases like ALS are expressed in all types of cells, even fibroblasts. Moreover, in *Mitochondria Health and Longevity* research group experiments results have supported that idea. Also, some researches have been able to obtain neurons from fibroblast reprogramming them, so as to use them as a treatment for MN diseases.[52,53]

1.3. Cellular pathway modulators

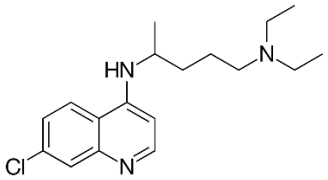
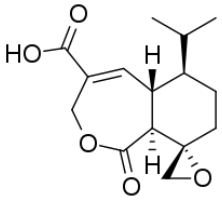
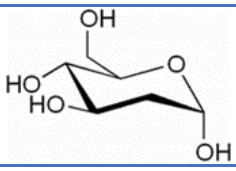
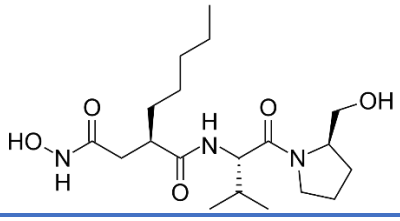
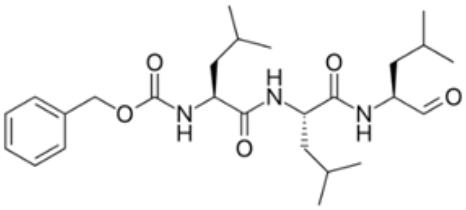
In the search of both cellular pathways and specific malfunctioning proteins that may be involved in the development of sALS, different chemical compounds known to modulate key cellular processes (*Table 7*) were added to both healthy and ALS patient cells, and the effect on protein expression level was analyzed. From now on, the cellular pathway modulators are going to be also referred as treatments, as some of them have been analyzed for the treatment of ALS or even cancer therapeutics.

On the one hand, Chloroquine (CLQ) the most widely used chemical which inhibits the last stage of the autophagy. However, the mechanism remains unknown, but as CLQ is a weak base, is thought to raise cellular pH and like that inhibit the process. Is also reported that CLQ severely affects the endo-lysosomal system and the Golgi complex in vitro and in vivo, impairing the basal autophagic flux by decreasing autophagosome-lysosome fusion, and inhibiting the degradation process.[54] Regarding MG132, also affects cell degradation, but instead of inhibiting autophagy, inhibits the proteasomal degradation. This is a peptide-aldehyde proteasome inhibitor that inhibits 20S proteasome activity by binding covalently to the active site of the beta sub-unit and blocking effectively the proteolytic activity of the 26S proteasome complex.[55]

The next two treatments are related to glycolysis and they inhibit different stages of this process. In the case of 2-Deoxy-d-glucose (2DG), this is a competitive inhibitor, this is, when enters into the cell competes with glucose and can inhibit glucose transport. When replaces glucose, 2DG is phosphorylated by hexokinase II to 2-deoxy-d-glucose-6-phosphate (2DG-6-P) but, this cannot be metabolized by phosphoglucose isomerase (PGI) to a 5-carbon ring. This ends in an accumulation of 2DG-6-P within the cell, allosteric and competitive inhibition of hexokinase, isomerase depletion of ATP, cell cycle detention and inhibition of cell growth, and thus, cell death. Therefore, the greater the amount of accumulated 2DG-6-P, the greater the effect on glycolysis.[56] However, 2DG has an off-target effect that must be taken into account, the inhibition of N-glycosylation of proteins.[57] Regarding Koningic Acid (KA), this is a lactone initially identified as an antimicrobial agent, active against anaerobic bacteria and displays antiparasitic properties. As to its effect in glycolysis, KA is an effective and specific inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), it inactivates GAPDH binding in a covalent way to a cysteine residue in the enzyme active part, which affects the synthesis of ATP.[58]

Apart from that, Actinonin is also used the one inhibits mitochondrial translation and in hence cellular breathing, which arrests cellular proliferation. Specifically induces a time-dependent loss of both the mitochondrial 12S and 16S rRNA, within the loss of mito-ribosomal subunits. Moreover, also happens a depletion of mitochondrial mRNA transcripts from both the light strand and heavy strand.[59,60]

Table 7. Chemical compounds administered and their cellular effect/function.

Treatments	Chemical structure	Function
Chloroquine		Autophagy last stage inhibitor.
KA		Glycolysis, GAPDH inhibitor.
2DG		Glucose competitive inhibitor.
Actinonin		Inhibits cell proliferation and mitochondrial translation.
MG132		Inhibition of the 26S complex of the proteosome.

Cells were incubated with the appropriate amount of the five modulators as monotherapy, and a sixth experiment was also carried out were Act and KA were coadministered. The incubation of both healthy and ALS patient cells with this cellular process modulators, and the analysis of their differential effect on the expression level of key proteins, was also used as a source of information with respect to possible origins of sALS.

1.4. Goals of the project

The goal of this project is to identify the cause of sporadic ALS or, if not the cause, a common protein expression pattern that may be related to this disease.

Our strategy consists of analyzing the differential protein expression level between healthy and ALS patient cells, proteins involved in the key cellular pathways mentioned before, ISR, protein degradation and dysfunction in glucose metabolism. Also, a second strategy to reach the goal of the project will be to analyze the effect of differ cellular process modulators (treatments) in differential protein expression of both type of patients. The main tool used for such purposes was Western blot (WB) analysis. We are aware that WB is a semiquantitative technique, and, therefore, the results obtained in different gels should not be compared quantitatively between them. Protein expression levels were consequently compared only inside the same gel and with the same amount of sample loaded in each line.

Regarding ISR, the UPR pathway has been related to the cause of cell death. So, first we should discard UPR as key in disease development by analyzing the proteins related with the pathway by WB analysis and confirm ISR as key in disease by confirming differences between healthy volunteers and patients in the expression of proteins reported by others as related in ALS. Talking about protein degradation, the malfunction of those pathways can aid the disease development, and not only that, the over/under expression of those proteins can also provide interesting information. Lastly, as a need of energy and malfunction of glycolysis has been related to ALS is also important to have in mind and make a hypothesis about this pathway, even if the evidences we have are very little.

This project was developed at the BioDonostia Health Research Institute (HRI), the first institute for health and research in the Basque Country, and one of the main institutes in which Osakidetza (the Basque Health Service, SVS) performs its research and innovation activities. It was created in 2008 and in 2011 was accredited as a Health Research Institute by the prestigious Carlos III Institute of Health. Since then, remains focused on the patient, improving people's health through its multi-institutional and inter-disciplinary collaborative structure. Generally, all kinds of samples are obtained from the Basque Biobank, mainly oncological and neurological tissues and biological blood samples are collected from diagnostic excess, or requested for research purposes, such us, cells, DNA, etc. to obtain information from different diseases.

2. MATERIALS AND METHODS

2.1. Cells and reagents.

Cellular samples were obtained from different volunteers. Three healthy patient cell biopsies (C or C1, C2 and C3) and three ALS patient cell biopsies (A or A1, A2 and A3) were conveniently managed through the study. Both control and disease samples consist of fibroblasts that were obtained by skin biopsies in BioDonostia by following an optimized protocol. This protocol is based on the next steps: First, we store the skin biopsies recently made in a transport medium. Then, we take out the fat, continued by cutting the skin in different little pieces and letting it dry in a flask, to provide the stuck on it. Finally, we add new medium so as fibroblast to start going out from the skin. Since then, each three days medium is changed and in a period of one month, the desired cells are obtained.

Reagents and medium used for the cell cultivation were the following: DMEM (Dulbecco's Modified Eagle Medium), with a Phenol red indicator, Sodium pyruvate additive and glutamine, with the addition of 5 mL GlutaMax (x100), 50 mL Feta Bovine Serum (FBS) (10 %) and Penicillin Streptomycin (P/S), Dulbecco's Phosphate Buffered Saline (dPBS), used for cell washing during the cultivation procedure, Trypsin, to provide de detachment of the cells from the flask and PBS/FBS, once cells are detached, is added to void the function of trypsin.

2.2. Cell culture

Cell culture was performed during various weeks, till the needed number of cells was obtained. The procedure is carried out each 2-3 days and is based on changing the cells from one flask to other and changing the medium. First, the medium is extracted from the flask and dPBS is added, this buffer takes the cells to a stand by situation, stopping its metabolism. After that, dPBS is extracted and trypsin is added and the flask taken to the incubator (37 ºC) to provide the detachment of the cells. Then, PBS/FBS must be added to cancel trypsin effect. Next, is taken to a falcon and centrifugated. Finally, the cells are divided into two new flasks with new media.

2.3. Sample types

As mentioned before, samples are obtained by the cultivation of fibroblasts. We have 3 sALS cell-lines and other 3 control lines. Apart from that, the experiments carried out were two and many WB were done making use of the protein obtained in those experiment. The first experiment is based on a single sALS cell-line and other control line, each with 5 different treatments (CLQ, KA, 2DG, Actinonin, KA+Actinonin), whereas the second is based on 3 sALS cell-lines and 3 control lines with a single treatment. However, in the second experiment two different treatments were applied MG132 and CLQ. For the better understanding of the plots, samples were named as follows: C stands for control and A stands for ALS patient samples.

2.4. Western Blot

WB was carried out following the protocol mentioned in the materials and methods chapter. Once proteins are lysed and prepared for the quantification, all aliquoted with the proteins of each cell-line and corresponding treatments are conserved in the refrigerator (-18 ºC). Once we start with the experimental part, the protein is charged in the gel and electrophoresis is executed for 90', followed by an overnight transfer from the gel to the membrane. After that blocking is carried out so as to provide the attachment of the 1º Ab in the protein and after that, washing and 2º Ab is added for 2h. Finally, membranes are revealed and protein is quantified by the iBright software programme. All results obtained by WB related to the

processes of interest during summer practices and 2021-22 curse are going to be analysed in the following chapters.

2.5. Sample Treatment

CLQ, KA, 2DG, Act and MG132 treatment solutions were acquire from stock solutions. For the acute treatments, cells were grown to 50–60% confluent and treated for 24 or 48 h with the protein expression modulators mentioned before (CLQ, KA, 2DG, Actinonin and KA+Actinonin). Treatments were added as indicated below: CLQ, 1000x, 50 µL to 10 mL medium, final concentration is 5 µM, MG132, 1000x, 50 µL to 10 mL, final concentration is 5 µM, KA, 1000x, 10 µL to 10 mL, final concentration is 1 µM, 2DG, 10x, 1 mL 2DG + 9 mL medium, final concentration is 10 mM, Act, 500x, 20 µL in 10 mL, the final concentration is1 µM and KA +Act, 10 µL KA and 20 µL act. in 10 mL, 1 µM KA and 1 µM Act.

2.6. Cell digestion and Western blot and Ab

To perform the Western Blot (WB) experiments the protocol was strictly followed. Cells were first lysed on ice in PBS, n-dodecyl-D-maltoside (DDM), 1X protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Abcam) and 50 Units Benzonase (Millipore). Protein concentration was determined by BCA RAPID GOLD kit (Thermofisher). Protein samples were prepared in 1× Laemmli loading buffer, heated at 42°C for 15 minutes and resolved on SDS-PAGE gels (Novex, Thermofisher Scientific). After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (PVDF, Millipore), previously activated with MeOH. Membranes were blocked with BSA in PBS with 0.1% (v/v) Tween-20 (PBST) and incubated overnight at 4°C with the primary antibodies in a 1:1000 proportion.

After the incubation, primary antibodies were washed in PBST and secondary antibodies were added in a 1:5000 proportion, chemiluminescence or fluorescent depending on the program that is going to be used to identify the protein. Then, the antibodies are going to be washed again and the developer West Pico PLUS (Thermos Fisher Scientific) will be added. Finally, the proteins will be revealed using iBright Imaging Systems (Thermos Fisher Scientific) and quantified using the iBright Analysis software programme.

2.7. Quantification of absolute and differential protein expression levels

Protein expression levels on healthy volunteers and ALS patients were analysed by WB, and absolute expression levels and relative or differential expression levels are reported below both before and after treatment. Differential expression levels are presented as % values, and they represent either over (+ values) or under expression (- values) of the protein under study in ALS patients with respect to controls, and were calculated according to equation 1:

$$\% = \frac{Absolute\ protein\ expression\ in\ ALS\ patients}{Absolute\ protein\ expression\ in\ healthy\ volunteers}\ x\ 100 - 100 \qquad \textit{Equation 1}$$

Therefore, positive differential expression values are related to overexpression of a specific protein in ALS patients as compared to healthy volunteers, whereas negative differential expression values are related to underexpression of a specific protein in ALS patients as compared to healthy volunteers.

As WB is a semiquantitative technique, the results obtained in different gels cannot be compared quantitatively between them. Anyway, different controls and ALS analysed in the same gels have been described with the standard deviation, taken into account that each ALS case probably will not have much common factor with the others.

3. RESULTS

Through this section, the protein expression level on 8 different samples will be analysed: four ALS cell-lines (A, A1, A2, A3, where A and A1 are from the same cell-line but A1 is a higher passage) and four Healthy volunteer (control) cell-lines: (C, C1, C2, C3, where C and C1 are the same cell-line but C1 is a higher passage). Two different experiments were carried out, the first is an n=1 experiment and is based on C vs. A, moreover, to compare the modulation of the cellular pathways, up to five different treatments (T) where added: T1: CLQ, T2: KA, T3: 2DG, T4: Act, T5: KA+Act. Whereas in the other experiment n=3 and is based on 3 C (C1, C2, C3) vs. 3 A (A1, A2, A3), but just with a single treatment that can be CLQ or MG132 respectively, referred as (T).

So, the samples were named as follows: for the first experiment CUT and AUT, for healthy (C) and ALS (A) cell-lines without incubation with cellular modulators, i.e., untreated (UT); CT1, CT2, CT3, CT4 and CT5 therefore, stands for healthy (C) cellular samples with incubation with cellular modulators, i.e., treated (T). In a similar fashion, AT1, AT2, AT3, AT4 and AT5 stand for ALS (A) patient derived cellular samples with incubation treated (T), with cellular pathway modulators. For the second experiment, C1UT, C2UT, C3UT and A1UT, A2UT, A3UT for healthy (C) and ALS (A) cell-lines for the untreated and C1T, C2T, C3T and A1T, A2T and A3T for healthy (C) and ALS (A) treated cell-lines. In the case of the second experiment samples, they are going to be described and plotted as with the media of the three samples and its standard deviation.

3.1. Checking the ISR pathway

Below is found the WB results obtained for key proteins implicated in the ISR pathway. The proteins analysed were eIF2α. CHOP, DELE1 and BIP. As mentioned in the introduction, the first two proteins are expressed by both UPR and heme depletion pathways, so the overexpression of DELE1 and the underexpression of BIP would confirm that contrary to UPR, heme depletion pathway has to do with sALS.

First common proteins are going to be analysed so as to confirm the activation of the ISR cascade, and then both results of BIP and DELE1 expression obtained is going to be observed so as to conclude some hypothesis about the specific cascade implicated in sALS.

Obtained quantification results for each protein are plotted in the graphs that are shown in each section. That data was obtained by WB technique mentioned before and by revealing the membrane and by normalizing it in the iBright software. One of the WB membrane results is the one that can be seen in *Figure 7*, for more results see annexes.

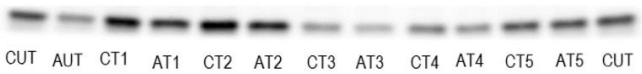


Figure 7. Revealed eIF2αP protein WB membrane.

Expression level of eIF2αP protein: Eukaryotic Translation Initiation Factor 2

Expression levels of Eukaryotic Translation Initiation Factor 2 protein (eIF2αP) quantified through WB analysis are found in *Figure 8* and *Table 8*. Represented data are averaged values obtained with several healthy volunteers (C) and ALS patients (A), before (UT) and after treatment (T) with the corresponding inhibitor/drug (For the first experiment: T1: CLQ, T2: KA, T3: 2DG, T4: Act, T5: KA+ACT and for the second T: MG132 or CLQ, it will be shown in the title of the graph).

Graphical analysis of the averaged absolute expression levels of eIF2αP protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs is found in Figure 8 and average numerical analysis of the results plotted is found in *Table 8*. As a reminder, differential expression levels are shown as percentages. Positive percentage values, such as %58, implies that the expression level of a specific protein is higher, in a 58%, in ALS patients, as compared to healthy patient. In the same line, negative percentage values such as -39,5%, implies that the expression level of a specific protein is lower, in a 39,5%, in ALS patients, as compared to healthy patients. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments, whereas T, in this particular case, referred to MG132.

The results show in *Figure 8* left, are the values obtained after performing a single experiment with each sample (n=1), whereas the results shown in *Figure 8* right, include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). All the details, including the WB analysis and additional experiments, have been omitted in the main text, but the reader is invited to analyze them in the Annexes (page 72).

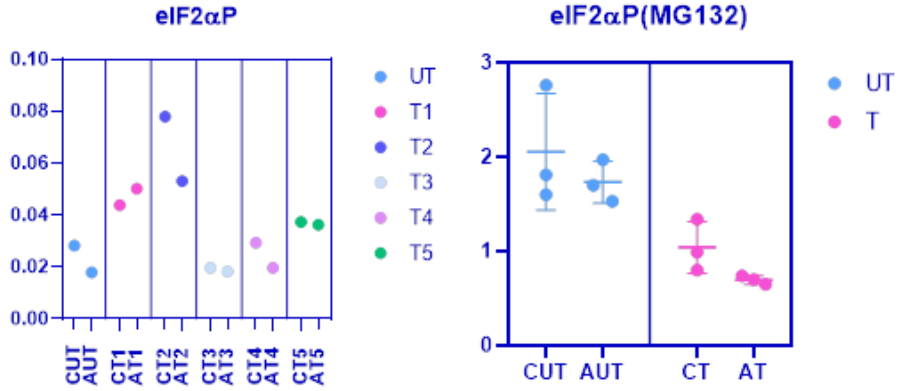


Figure 8. *Graphical representation (of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of eIF2αP protein.*

Table 8. *Numerical analysis of the absolute and differential eIF2αP expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
INNER STRESS RESPONSE							
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
eIF2αP Anexe_WB2	1,85	0,920	-50,3%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	2,55 3,01 1,14 1,59 2,14	4,04 3,75 0,69 1,13 1,95	58,0% 24,0% -39,5% -28,9% -8,90%
eIF2αP Anexe_WB8	0,0283	0,018 0	-36,4%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	0,0439 0,0780 0,0197 0,0294 0,0374	0,0502 0,0531 0,0183 0,0197 0,0363	14,0% -31,9% -7,10% -33,0% -3,00%
eIF2αP Anexe_WB9	2,06± 0,62	1,73± 0,22	-16,0%	MG132 (T)	1,04±0,27	0,697±0,026	-33,0%

As concluded from *Figure 8* and *Table 8*:

a) The values of the absolute and relative expression levels of eIF2αP protein before treatment indicate that eIF2αP is significantly underexpressed in ALS patients with respect to healthy volunteers, with an expression level of eIF2αP of a -50,3% in the former with respect to the latter. In the case of the second experiment can be seen that is just moderately underexpressed (-16,0%), but in general, we can say that before treatment sALS cell-lines are at least moderately underexpressed compared to control cells.

b) The values of the absolute and relative expression levels of eIF2αP protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of eIF2αP is moderately affected in the case of Healthy volunteers, with the induction of expression of eIF2αP of a 37,8%. In the same line, expression level of eIF2αP is importantly affected by this treatment in case of ALS patients, with the induction of expression of eIF2αP of a 339%.

  The final differential expression of eIF2αP protein after treatment with CLQ was therefore significant since CLQ triggered an increase of eIF2αP protein expression of a 58,0% in ALS patients with respect to healthy patients. In the other WB obtained with the same first experiment samples same tendency is obtained, even if it is seen moderately.

- Upon treatment with KA expression level of eIF2αP is significantly affected in the case of Healthy volunteers, with the induction of expression of eIF2αP of a 62,7%. In the same line, expression level of eIF2αP is importantly affected by this treatment in case of ALS patients, with the induction of expression of eIF2αP of a 308%. This can be also seen in the other WB done with the same samples.

  The final differential expression of eIF2αP protein after treatment with KA was therefore significant since KA triggered an increase of eIF2αP protein expression of a 58% in ALS patients with respect to healthy patients. In this case, in the second WB cannot be seen the same differential expression, as the increase of expression is higher in healthy cells than in sALS cells.

- Upon treatment with 2DG expression level of eIF2αP is moderately affected in the case of Healthy volunteers, with the inhibition of expression of eIF2αP of a -38%. In the same line, expression level of eIF2αP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of eIF2αP of a -25%.

  The final differential expression of eIF2αP protein after treatment with 2DG was therefore moderate, since 2DG triggered a decrease of eIF2αP protein expression of a -39,5 in ALS patients with respect to healthy patients. The same is seen in the second WB membrane, but in an even more moderate way.

- Upon treatment with Act expression level of eIF2αP is moderately affected in the case of Healthy volunteers, with the inhibition of expression of eIF2αP of a -14,0%. In the same line, expression level of eIF2αP is moderately affected by this treatment in case of ALS patients, with the induction of expression of eIF2αP of a 22,8%.

  The final differential expression of eIF2αP protein after treatment with Act was therefore moderate, since Act triggered a decrease of eIF2αP protein expression of a -28,9% in ALS

patients with respect to healthy patients. In the second WB the total protein expression is similar -33,0%, but after treatment healthy cell-line protein expression is partially induced.

- Upon treatment with KA+Act expression level of eIF2αP is moderately affected in the case of Healthy volunteers, with the induction of expression of eIF2αP of a 15,7%. In the same line, expression level of eIF2αP is importantly affected by this treatment in case of ALS patients, with the induction of expression of eIF2αP of a 112%.

  The final differential expression of eIF2αP protein after treatment with KA+Act was therefore almost insignificant, since KA+Act triggered a decrease of eIF2αP protein expression of a -8,90% in ALS patients with respect to healthy patients. In the second WB can also be seen an increase in expression and an underexpression in total protein expression.

- Upon treatment with MG132 expression level of eIF2αP is significantly affected in the case of Healthy volunteers, with the inhibition of expression of eIF2αP of a -49,5%. In the same line, expression level of eIF2αP is significantly affected by this treatment in case of ALS patients, with the inhibition of expression of eIF2αP of a -59%.

  The final differential expression of eIF2αP protein after treatment with MG132, was therefore significant, since MG132 triggered a decrease of eIF2αP protein expression of a -33,0% in ALS patients with respect to healthy patients.

We can observe that in all cases before treatment, ALS has a lower concentration of eIF2αP than healthy volunteers. Moreover, the inhibition of the autophagy (CLQ) increases protein expression, whereas when inhibiting the proteosome eIF2αP protein expression decreases. With actinonin an increase can be seen as its effect produces a stress situation. In the case of KA and 2DG a contrary effect is created, the first increases protein expression, when the second inhibits eIF2αP.

Expression level of CHOP protein: C/EBP Homologous protein transcription factor

Expression levels of C/EBP Homologous protein transcription factor (CHOP) quantified through WB analysis are found in *Figure 9* and *Table 9*. The graphical analysis of the averaged absolute expression levels of CHOP protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments, whereas T, in this particular case, is referred to MG132.

As in the case above, the results show in *Figure 9* left, are the values obtained after performing a single experiment with each sample (n=1), whereas the results shown in *Figure 9* right, include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). All the details, including the WB analysis and additional experiments, have been omitted in the main text, but the reader is invited to analyze them in the Annexes (page 73).

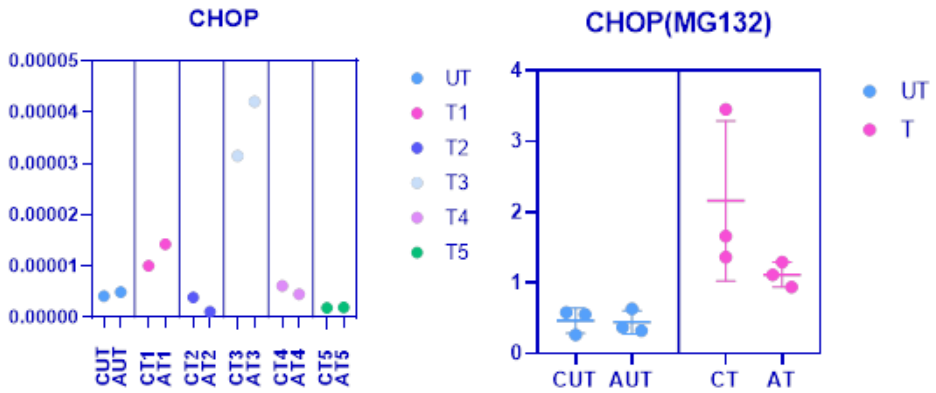


Figure 9. *Graphical representation (of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of CHOP protein.*

Table 9. *Numerical analysis of the absolute and differential CHOP expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
INNER STRESS RESPONSE							
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
CHOP Anexe_WB8	4,17E-06	4,98E-06	19,4%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	1,01E-05 3,93E-06 3,15E-05 6,17E-06 1,88E-06	1,43E-05 1,16E-06 4,21E-05 4,58E-06 1,97E-06	41,6% -70,5% 33,6% -25,8% 4,79%
CHOP Anexw_WB1	4,07E-06	1,31E-05	222%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	5,29E-06 3,36E-06 1,12E-05 6,32E-06 5,85E-06	7,47E-06 1,32E-05 2,96E-05 2,29E-05 2,35E-05	41,2% 293% 164% 262% 302%
CHOP Anexe_WB2	1,60	1,94	21,2%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	1,45 1,83 2,87 1,95 3,05	3,14 2,96 2,14 2,33 3,41	116% 61,7% -25,4% 19,5% 11,8%
CHOP Anexe_WB9	0,463± 0,177	2,16± 1,13	336%	MG132 (T)	0,440± 0,096	1,11± 0,10	152%

As concluded from *Figure 9* and *Table 9*:

a) The values of the absolute and relative expression levels of CHOP protein before treatment indicate that CHOP is moderately overexpressed in ALS patients with respect to healthy patients, with an expression level of CHOP of a 19,4% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of CHOP protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of CHOP is importantly affected in the case of Healthy volunteers, with the induction of expression of CHOP of a 142%. In the same line,

expression level of CHOP is importantly affected by this treatment in case of ALS patients, with the induction of expression of CHOP of a 187%.

The final differential expression of CHOP protein after treatment with CLQ was therefore moderate, since CLQ, triggered an increase of CHOP protein expression of a 41,6% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of CHOP is almost non affected in the case of Healthy volunteers, with the inhibition of expression of CHOP of a -5,76%. In the same line, expression level of CHOP is significantly affected by this treatment in case of ALS patients, with the inhibition of expression of CHOP of a -76,7%.

  The final differential expression of CHOP protein after treatment with KA was therefore significant, since KA triggered a decrease of CHOP protein expression of a -70,5% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of CHOP is importantly affected in the case of Healthy volunteers, with the induction/inhibition of expression of CHOP of a 655%. In the same line, expression level of CHOP is importantly affected by this treatment in case of ALS patients, with the induction/inhibition of expression of CHOP of a 745%.

  The final differential expression of CHOP protein after treatment with 2DG was therefore moderate, since 2DG triggered an increase of CHOP protein expression of a 33,6 in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of CHOP moderately affected in the case of Healthy volunteers, with the induction of expression of CHOP of a 48,0%. On the contrary, expression level of CHOP is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of CHOP of a -8,03%.

  The final differential expression of CHOP protein after treatment with Act was therefore moderate, since Actinonin triggered a decrease of CHOP protein expression of a -25,8% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of CHOP is moderately affected in the case of Healthy volunteers, with the inhibition of expression of CHOP of a -54,9%. In the same line, expression level of CHOP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of CHOP of a -60,4%

  The final differential expression of CHOP protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered an increase of CHOP protein expression of a 4,79% in ALS patients with respect to healthy patients.

- Upon treatment with MG132 expression level of CHOP is almost non affected in the case of Healthy volunteers, with the inhibition of expression of CHOP of a -4,97%. In the same line, expression level of CHOP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of CHOP of a -48,6%.

  The final differential expression of CHOP protein after treatment with MG132, was therefore significant, since MG132 triggered an increase of CHOP protein expression of a 152% in ALS patients with respect to healthy patients.

We can observe that in all WBs obtained, ALS cell-lines have a higher concentration of CHOP. Regarding the effect of treatments, we can see some different results with the same treatments in the total protein expression.

Expression level of DELE1 protein: DAP3 Binding Cell Death Enhancer 1

Expression levels of DAP3 Binding Cell Death Enhancer 1 (DELE1) quantified through WB analysis are found in *Figure 10* and *Table 10*. The graphical analysis of the averaged absolute expression levels of DELE1 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. A single T is applied, in this particular case, is referred to MG132.

The results show in *Figure 10* include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). All the details, including the WB analysis and additional experiments, have been omitted in the main text, but the reader is invited to analyze them in the Annexes (page 74-75).

As it is mentioned in the introduction, DELE1 is split by OMA1 and in hence we have two DELE1 units, that are $DELE1_L$ and $DELE1_S$ which activates HRI. However, in the results obtained in the laboratory three bands were observed, not only two as in the articles reported lately. So, we decided to name three different units: $Dele1_L$, $DELE1_{SA}$ and $DELE1_{SB}$, supposing that can happen an unspecific cut and we could have different $DELE1_S$.

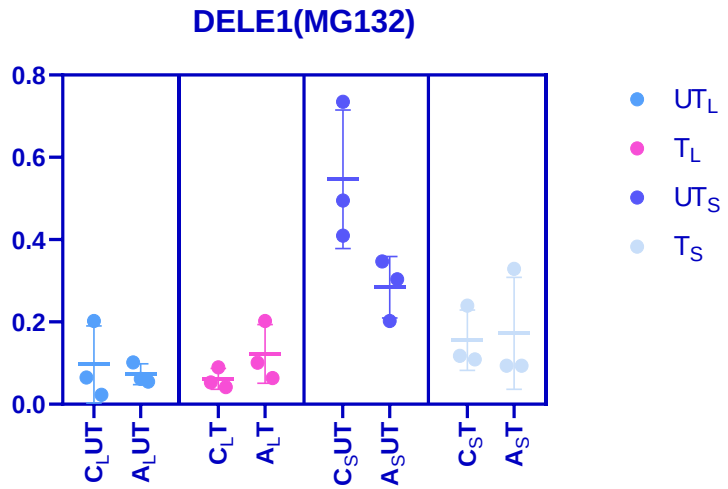


Figure 10. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of DELE1 protein.*

Table 10. *Numerical analysis of the absolute and differential DELE1 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**HEME DEPRIVATION**						
Protein	**Healthy**	**ALS**	**ALS vs. Helathy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
$DELE1_L$ Anexes_WB3	1,45E-01	7,45E-02	-48,6%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	9,56E-02 6,54E-02 4,78E-02 5,77E-02 2,12E-01	1,95E-02 5,08E-02 9,83E-03 3,82E-02 4,27E-02	-79,6% -22,3% -79,4% -33,8% -79,8%
$DELE1_{SA}$ Anexes_WB3	0,287	0,684	138%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	0,253 0,162 0,252 0,066 0,259	0,134 0,188 0,519 0,495 0,840	-47,0% 16,0% 106% 650% 224%
$DELE1_{SB}$ Anexes_WB3	0,287	0,612	113%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	0,180 0,079 0,109 0,142 0,386	0,321 0,331 0,327 0,476 0,626	78,3% 319% 200% 235% 62,2%
$DELE1_S$ Anexes_WB7	2,22± 0,74	1,70± 0,19	-23,4%	CLQ (T)	1,71± 0,77	2,57± 0,54	50,3%
$DELE1_L$ Anexes_WB9	0,0967± 0,0936	0,0730± 0,0254	-24,5%	MG132 (T)	0,0617± 0,0251	0,122± 0,0714	97.7%
$DELE1_S$ Anexes_WB9	0,547± 0,168	0,273± 0,094	-50,1%	MG132 (T)	0,156± 0,073	0,172± 0,136	10,2%

As concluded from *Figure 10* and *Table 10:*

a) The values of the absolute and relative expression levels of $DELE1_L$ protein before treatment indicate that $DELE1_L$ is moderately underexpressed in ALS patients with respect to healthy patients, with an expression level of $DELE1_L$ of a -48,6% in the former with respect to the latter.

The values of the absolute and relative expression levels of $DELE1_S$ protein before treatment indicate that $DELE1_S$ is significantly overexpressed in ALS patients with respect to healthy patients, with an expression level of $DELE1_S$ of a 138% in the case of $DELE1_{SA}$ and 113% in the case of $DELE1_{SB}$ in the former with respect to the latter. In the case of other experiments, we can see that before treatment in ALS is an underexpression of $DELE1_S$ of a -23,4% and -50,1%.

b) The values of the absolute and relative expression levels of $DELE1_L$ protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of $DELE1_L$ is moderately affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_L$ of a -34,1%. In the same line, expression level of $DELE1_L$ is significantly affected by this treatment in case of ALS patients, with the induction/inhibition of expression of $DELE1_L$ of a -73,8%.

  The final differential expression of $DELE1_L$ protein after treatment with CLQ was therefore significant, since CLQ triggered a decrease of $DELE1_L$ protein expression of a -79,6% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of $DELE1_L$ is moderately affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_L$ of a -54,9%. In the same line, expression level of $DELE1_L$ is moderately affected by this treatment in case of ALS patients, with the induction/inhibition of expression of $DELE1_L$ of a -31,8%.

  The final differential expression of $DELE1_L$ protein after treatment with KA was therefore moderate, since KA triggered a decrease of $DELE1_L$ protein expression of a -22,3% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of $DELE1_L$ is significantly affected in the case of Healthy volunteers, with the induction/inhibition of expression of $DELE1_L$ of a -67,0%. In the same line, expression level of $DELE1_L$ is significantly affected by this treatment in case of ALS patients, with the inhibition of expression of $DELE1_L$ of a -86,8%.

  The final differential expression of $DELE1_L$ protein after treatment with 2DG was therefore significant, since 2DG triggered a decrease of $DELE1_L$ protein expression of a -79,8% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of $DELE1_L$ is significantly affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_L$ of a -60,2%. In the same line, expression level of $DELE1_L$ is moderately affected by this treatment in case of ALS patients, with the induction/inhibition of expression of $DELE1_L$ of a -48,8%.

  The final differential expression of $DELE1_L$ protein after treatment with Act was therefore moderate, since Act triggered a decrease of $DELE1_L$ protein expression of a -33,8% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of $DELE1_L$ is moderately affected in the case of Healthy volunteers, with the induction of expression of $DELE1_L$ of a 46,2%. On the contrary, expression level of $DELE1_L$ is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of $DELE1_L$ of a -42,7%.

  The final differential expression of $DELE1_L$ protein after treatment with KA+Act was therefore significant, since KA+Act triggered a decrease of $DELE1_L$ protein expression of a -79,8% in ALS patients with respect to healthy patients.

- Upon treatment with MG132 expression level of $DELE1_L$ is moderately affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_L$ of a -36,2%. On the contrary, expression level of $DELE1_L$ is significantly affected by this treatment in case of ALS patients, with the induction of expression of $DELE1_L$ of a 67,1%.

  The final differential expression of $DELE1_L$ protein after treatment with MG132 was therefore significant, since MG132 triggered an increase of $DELE1_L$ protein expression of a 97,7% in ALS patients with respect to healthy patients.

c) The values of the absolute and relative expression levels of $DELE1_S$ protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of $DELE1_{SA}$ is insignificantly affected in the case of Healthy volunteers, with the induction of expression of $DELE1_{SA}$ of a -11,8%. In the same line, expression level of $DELE1_{SA}$ is significantly affected by this treatment in case of ALS patients,

with the induction/inhibition of expression of $DELE1_{SA}$ of a -80,4%. Similarly happens with $DELE1_{SB}$. In the other WB, in healthy volunteers the same tendency can be seen, but in the case of ALS an important induction is observed.

The final differential expression of $DELE1_{SA}$ protein after treatment with CLQ was therefore moderate, since CLQ triggered a decrease of $DELE1_{SA}$ protein expression of a -47,0% in ALS patients with respect to healthy patients. The contrary happens in the case of $DELE1_{SB}$ that is significantly overexpressed with a protein expression of 78,3%. If we observe the other WB experiment, we can also see an increase in the total protein expression.

- Upon treatment with KA expression level of $DELE1_{SA}$ is moderately affected in the case of Healthy volunteers, with the induction/inhibition of expression of $DELE1_{SA}$ of a -45,6%. On the contrary/In the same line, expression level of $DELE1_{SA}$ is significantly by this treatment in case of ALS patients, with the inhibition of expression of $DELE_{SA}$ of a -72,5%. Similar tendencies can be seen in the expression of $DELE1_{SB}$.

  The final differential expression of $DELE1_{SA}$ protein after treatment with KA was therefore insignificant, since KA triggered an increase of $DELE_{SA}$ protein expression of a 16,0% in ALS patients with respect to healthy patients. In the case of $DELE1_{SB}$ same happens but with a much higher overexpression 319%.

- Upon treatment with 2DG expression level of $DELE1_{SA}$ is insignificantly affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_{SA}$ of a -12.2%. In the same line, expression level of $DELE1_{SA}$ is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of $DELE1_{SA}$ of a -15,2%. The same happens with $DELE1_{SB}$ but expression moderately inhibited.

  The final differential expression of $DELE1_{SA}$ protein after treatment with 2DG was therefore significant, since 2DG triggered an increase of $DELE1_{SA}$ protein expression of a 106% in ALS patients with respect to healthy patients. In the same way, $DELE1_{SB}$ protein expression is importantly induced a 200%.

- Upon treatment with Act expression level of $DELE1_{SA}$ is significantly affected in the case of Healthy volunteers, with the inhibition of expression of $DELE1_{SA}$ of a -77,0%. In the same line, expression level of $DELE1_{SA}$ is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of $DELE1_{SA}$ of a -27,6%. Same tendencies are observed in $DELE1_{SB}$.

  The final differential expression of $DELE1_{SA}$ protein after treatment with Act was therefore important, since Act triggered an increase of $DELE1_{SA}$ protein expression of a 650% in ALS patients with respect to healthy patients, same with $DELE1_{SB}$ as it is also induced in a 235%.

- Upon treatment with KA+Act expression level of $DELE1_{SA}$ is almost non affected in the case of Healthy volunteers, with the induction/inhibition of expression of $DELE1_{SA}$ of a -9,76%. On the contrary, expression level of $DELE1_{SA}$ is almost non affected by this treatment in case of ALS patients, with the induction of expression of $DELE1_{SA}$ of a 22,8%. In the case of $DELE1_{SB}$ in healthy volunteer a moderate induction can be seen but in the case of ALS is not affected.

  The final differential expression of $DELE1_{SA}$ protein after treatment with KA+Act was therefore important, since KA+Act triggered an increase of $DELE1_{SA}$ protein expression of a 224% in ALS

patients with respect to healthy patients. Same in the case of DELE1$_{SB}$ but with a moderate induction.

- Upon treatment with MG132 expression level of DELE1$_S$ is significantly affected in the case of Healthy volunteers, with the inhibition of expression of DELE1$_S$ of a -71,5%. On the contrary, expression level of DELE1$_S$ is significantly affected by this treatment in case of ALS patients, with the inhibition of expression of DELE1$_S$ of a -37,0%.

  The final differential expression of DELE1$_S$ protein after treatment with MG132 was therefore insignificant, since MG132 triggered an increase of DELE1$_S$ protein expression of a 10,2% in ALS patients with respect to healthy patients.

We can observe that in the case of DELE1$_L$ there is a smaller concentration in ALS cell-lines and it generally decreases after treatment. DELE1$_{SA}$ and DELE1$_{SB}$ follow the same tendencies, higher concentrations are quantified in ALS cells and generally decreases after treatment.

Expression level of BIP protein: Binding Immunoglobulin Protein

Expression levels of Binding Immunoglobulin Protein (BIP) quantified through WB analysis are found in *Figure 11* and *Table 11*.The graphical analysis of the averaged absolute expression levels of BIP protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. In the plot T makes reference to usual treatments, in this particular case, is referred to MG132.

The results show in Figure 11 include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). All the details, including the WB analysis and additional experiments, have been omitted in the main text, but the reader is invited to analyze them in the annexes (page 72).

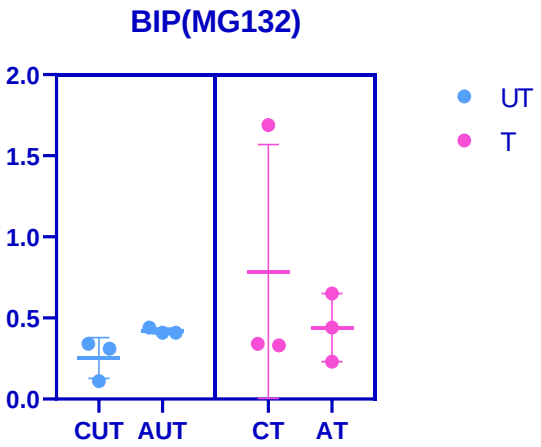


Figure 11. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of DELE1 protein.*

Table 11. *Numerical analysis of the absolute and differential BIP expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**ER STRESS (UPR)**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
BIP Anexe_WB1	9,27E-06	6,61E-06	-28,7%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	1,23E-05 7,74E-06 2,92E-05 8,34E-06 3,63E-06	5,28E-06 5,30E-06 2,40E-05 1,94E-06 4,17E-06	-57,1% -31,5% -17,8% -76,7% 14,9%
BIP Anexe_WB9	0,253± 0,125	0,420± 0,017	66,0%	MG132 (T)	0,787± 0,782	0,440± 0,210	-44,1%

As concluded from *Figure 11* and *Table 11:*

a) The values of the absolute and relative expression levels of BIP protein before treatment indicate that BIP is moderately underexpressed in ALS patients with respect to healthy patients, with an expression level of BIP of a -28,7% in the former with respect to the latter, but in the case of 3 ALS vs. 3 C, a moderately overexpression is seen with a 66,0%.

b) The values of the absolute and relative expression levels of BIP protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of BIP is moderately affected in the case of Healthy volunteers, with the induction of expression of BIP of a 32,7%. On the contrary, expression level of BIP is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of BIP of a -20,1%.

  The final differential expression of BIP protein after treatment with CLQ was therefore moderate, since CLQ triggered a decrease of BIP protein expression of a -57,1% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of BIP is insignificantly affected in the case of Healthy volunteers, with the inhibition of expression of BIP of a -16,5%. In the same line, expression level of BIP is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of BIP of a -19,8%.

  The final differential expression of BIP protein after treatment with KA was therefore moderate, since KA triggered a decrease of BIP protein expression of a -31,5% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of BIP is importantly affected in the case of Healthy volunteers, with the induction of expression of BIP of a 215%. In the same line, expression level of BIP is importantly affected by this treatment in case of ALS patients, with the induction of expression of BIP of a 263%.

  The final differential expression of BIP protein after treatment with 2DG was therefore insignificant, since 2DG triggered a decrease of BIP protein expression of a -17,8% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of BIP is almost non affected in the case of Healthy volunteers, with the inhibition of expression of BIP of a -10,0%. In the same line, expression level of BIP is significantly affected by this treatment in case of ALS patients, with the induction/inhibition of expression of BIP of a -70,6%.

  The final differential expression of BIP protein after treatment with Act was therefore significant, since Act triggered a decrease of BIP protein expression of a -76,7% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of BIP is moderately affected in the case of Healthy volunteers, with the induction/inhibition of expression of BIP of a -60,8%. In the same line, expression level of BIP is moderately affected by this treatment in case of ALS patients, with the induction/inhibition of expression of BIP of a -36,9%.

  The final differential expression of BIP protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered an increase of BIP protein expression of a 14,9% in ALS patients with respect to healthy patients.

- Upon treatment with MG132 expression level of BIP is importantly affected in the case of Healthy volunteers, with the induction/inhibition of expression of BIP of a 211%. On the contrary, expression level of BIP is not affected by this treatment in case of ALS patients, with the induction/inhibition of expression of BIP of a 4,76%.

  The final differential expression of BIP protein after treatment with MG132 was therefore moderate, since MG132 triggered a decrease of BIP protein expression of a -44,1% in ALS patients with respect to healthy patients.

We can observe that there are some incongruencies between both experiment results in the before treatment expression, as in the first ALS cells have a lower expression, whereas the contrary is seen in the second experiment.

3.2. Checking protein degradation pathways

Next is found the WB results obtained for key proteins implicated in the protein degradation pathways. The proteins analysed are Free Ub, Ub, UBQLN2, VCP, LC3B, P62 and CD147. Most proteins are related to both proteasomal and lysosomal degradation, but LC3B and P62 are just related to autophagy, whereas CD147 is related with the proteosome. Following the same structure, first common proteins are going to be analysed.

Expression level of Ub protein: Ubiquitin

Expression levels of Ubiquitin (Ub) quantified through WB analysis are found in *Figure 12* and *Table 12.* The graphical analysis of the averaged absolute expression levels of Ub protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments, whereas T, in this particular case, is referred to MG132.

The results show in *Figure 12* left, are the values obtained after performing a single experiment with each sample (n=1), whereas the results shown in *Figure 12* right, include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). All the details, including the WB analysis and additional experiments, have been omitted in the main text, but the reader is invited to analyze them in the annexes (page 76-77).

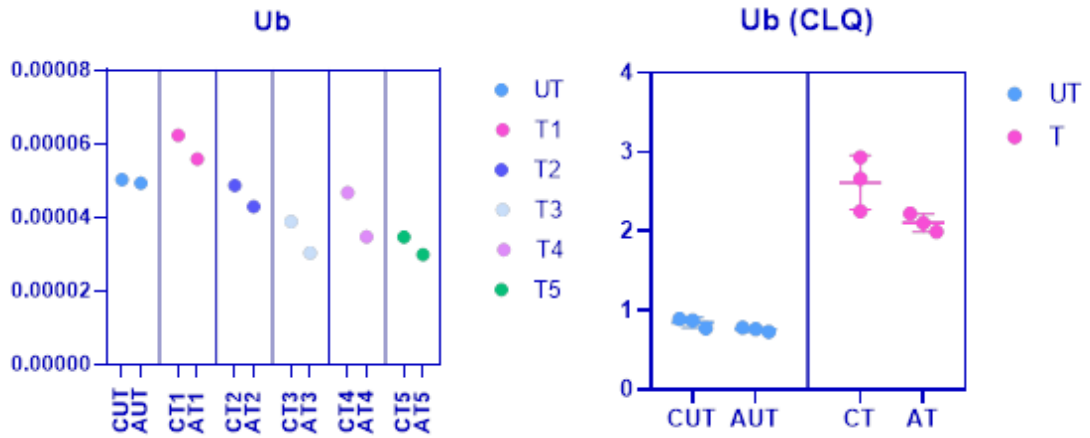


Figure 12. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of Ub protein.*

Table 12. *Numerical analysis of the absolute and differential Ub expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**PROTEIN DEGRADATION**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
Ub Anexe_WB9	5,02E-05	4,93E-05	-12,5%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	6,23E-05 4,86E-05 3,88E-05 4,67E-05 3,46E-05	5,68E-05 4,29E-05 3,03E-05 3,47E-05 2,98E-05	-8,83% -12,1% -21,9% -25,7% -13,9%
Ub Anexe_WB2	3,04	2,67	-12,2%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	2,97 1,91 1,77 2,30 1,92	4,84 2,26 1,48 2,11 2,05	63,0% 18,3% -16,4% -8,26% 6,77%
Ub Anexe_WB3	0,692	0,500	-27,7%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	0,668 0,694 0,674 0,638 0,771	0,780 0,610 0,531 0,459 0,682	16,8% -12,1% -21,2% -28,0% -11,5%
Ub Anexe_WB9	0,843± 0,062	0,758± 0,026	-10,1%	MG132 (T)	2,61± 0,34	2,10± 0,07	-19,5%
Ub Anexe_WB4	0,304± 0,013	0,552± 0,044	81,6%	CLQ (T)	0,376± 0,103	0,674± 0,042	79,2%
Ub Anexe_WB5	1,98± 0,24	2,56± 0,15	29,3%	CLQ (T)	1,95± 0,10	3,71± 0,57	90,2%
Ub Anexe_WB4	0,202± 0,045	0,218± 0,009	7,92%	MG132 (T)	0,782± 0,077	0,817± 0,090	4,48%

As concluded from *Figure 12* and *Table 12:*

a) The values of the absolute and relative expression levels of Ub protein before treatment indicate that Ub is generally insignificantly overexpressed or underexpressed, depending on the experiment, in ALS patients with respect to healthy patients, with an expression level of Ub of a ±10-30% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of Ub protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of Ub is almost non affected in the case of Healthy volunteers, with the induction or inhibition of expression of Ub of a ±3-10%, but in some experiments of n=3 samples, we can observe a bigger change of a 210%. On the contrary, expression level of Ub is significantly affected by this treatment in case of ALS patients, with the induction of expression of Ub of a 50-90%.

  The final differential expression of Ub protein after treatment with CLQ was generally significant, since CLQ triggered an increase of Ub protein expression of a 16-90% in ALS patients with respect to healthy patients, anyway this tendency cannot be observed in all experiments.

- Upon treatment with KA expression level of Ub is almost non affected in the case of Healthy volunteers, with the inhibition of expression of Ub of a -4-37%. On the contrary, expression level

of Ub is almost non affected by this treatment in case of ALS patients, with the induction/inhibition of expression of Ub of a ±15,0%.

The final differential expression of Ub protein after treatment with CLQ was therefore insignificant, since KA triggered an increase or decrease of Ub protein expression of a ±10-20% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of Ub is moderately affected in the case of Healthy volunteers, with the induction/inhibition of expression of Ub of around -40%. In the same line, expression level of Ub is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of Ub of a -50-80%.

  The final differential expression of Ub protein after treatment with 2DG was therefore insignificant, since 2DG triggered a decrease of Ub protein expression of a 10-20% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of Ub is insignificantly affected in the case of Healthy volunteers, with the inhibition of expression of Ub of around 10-20%. On the contrary, expression level of Ub is almost non affected by this treatment in case of ALS patients, with the induction/inhibition of expression of Ub of around -20%.

  The final differential expression of Ub protein after treatment with Act was therefore insignificant, since Act triggered a decrease of Ub protein expression of a -10-20% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of Ub is almost non affected in the case of Healthy volunteers, with the induction or inhibition of expression of Ub of a ±10%. In the same line, expression level of Ub is almost non affected by this treatment in case of ALS patients, with the induction/inhibition of expression of Ub of a ±20%.

  The final differential expression of Ub protein after treatment with KA+Act was therefore insignificant, since KA triggered an increase or decrease of Ub protein expression of a ±10% in ALS patients with respect to healthy patients.

- Upon treatment with MG132 expression level of Ub is importantly affected in the case of Healthy volunteers, with the induction of expression of Ub of a 287%. In the same line, expression level of Ub is importantly affected by this treatment in case of ALS patients, with the induction of expression of Ub of a 275%.

  The final differential expression of Ub protein after treatment with MG132 was therefore insignificant, since MG132 triggered an increase or of Ub protein expression of a 4,48% in ALS patients with respect to healthy patients.

We can observe that not all experiments accord between them. CLQ affect in a higher way to sALS cells. As it was expected MG132 have a high effect on Ub expression, with an induction.

Expression level of Free Ub protein: Free Ubiquitin

Expression levels of Free Ubiquitin (Free Ub) quantified through WB analysis are found in *Figure 13* and *Table 13*. The graphical analysis of the averaged absolute expression levels of Free Ub protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments. The results show in Figure 13, are the values obtained after performing a single experiment with each sample (n=1). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 82).

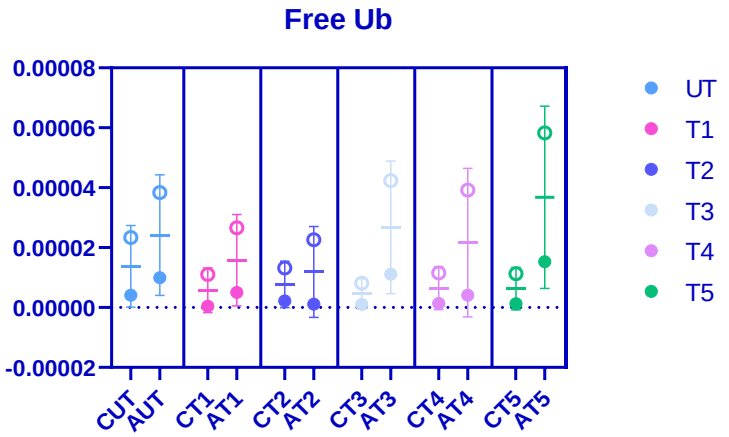


Figure 13. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of Free Ub protein.*

Table 13. *Numerical analysis of the absolute and differential Free Ub expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**PROTEIN DEGRADATION**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
Free Ub (Band1) Anexe_WB1	4,15E-06	9,95E-06	140%	CLQ (T1)	4,64E-07	5,00E-06	978%
				KA (T2)	2,20E-06	1,14E-06	-48,2%
				2DG (T3)	1,02E-06	1,11E-05	988%
				Act (T4)	1,41E-06	4,16E-06	195%
				KA+Act (T5)	1,26E-06	1,53E-05	1114%
Free Ub (Band2) Anexe_WB1	2,34E-05	3,84E-05	64,1%	CLQ (T1)	1,10E-05	2,66E-05	142%
				KA (T2)	1,32E-05	2,26E-05	71,2%
				2DG (T3)	8,13E-06	4,24E-05	422%
				Act (T4)	1,15E-05	3,92E-05	241%
				KA+Act (T5)	1,13E-05	5,83E-05	416%

As concluded from *Figure 13* and *Table 13:*

a) The values of the absolute and relative expression levels of Free Ub protein before treatment indicate that Free Ub is importantly for band 1 and moderately for band 2 in ALS patients with respect to healthy patients, with an expression level of Free Ub of a 140% for band 1 and a 64,1% for band 2 in the former with respect to the latter.

b) The values of the absolute and relative expression levels of Free Ub protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of Free Ub is significantly affected in the case of Healthy volunteers, with the inhibition of expression of Free Ub of a -88,8%.and a -53,0% in the case of band 1 and band 2 respectively. In the same line, expression level of Free Ub is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of Free Ub of a -49,7% and a -30,7% for band 1 and band 2 respectively.

  The final differential expression of Free Ub protein after treatment with CLQ was therefore important for band 1 and significant for band 2, since CLQ triggered an increase of Free Ub protein expression of a 978% for band 1 and 142% for band 2 in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of Free Ub is moderately affected in the case of Healthy volunteers, with the inhibition of expression of Free Ub of a -47,0% for band 1, similar happens with band 2. In the same line, expression level of Free Ub is significantly affected by this treatment in case of ALS patients, with the inhibition of expression of Free Ub of a -88,5%. Same happens with band 2 but moderately.

  The final differential expression of Free Ub protein after treatment with KA was therefore moderate, since KA triggered a decrease of Free Ub protein expression of a -48,2% in the case of band 1. The contrary happens in the case of ban 2, where Free Ub is significantly induced with a 71,2% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of Free Ub is affected in the case of Healthy volunteers, with the inhibition of expression of Free Ub of a -75,4% for band 1 and -65,2% for band 2. On the contrary, expression level of Free Ub is almost non affected by this treatment in case of ALS patients, with the induction of expression of Free Ub of a 11,6%.

  The final differential expression of Free Ub protein after treatment with 2DG was therefore important, since 2DG triggered an increase of Free Ub protein expression of a 988% for band 1 and 422% for band 2 in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of Free Ub is moderately affected in the case of Healthy volunteers, with the inhibition of expression of Free Ub of a -66,0%, similar happens in both bands. In the same line, expression level of Free Ub is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of Free Ub of a -58,2%, similar for both bands.

  The final differential expression of Free Ub protein after treatment with Act was therefore important, since Act triggered an increase of Free Ub protein expression of a 195% and 241% for band 1 and band 2 respectively in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of Free Ub is moderately affected in the case of Healthy volunteers, with the inhibition of expression of Free Ub of a -69,6%, similar in both bands. On the contrary, expression level of Free Ub is moderately affected by this treatment in case of ALS patients, with the induction of expression of Free Ub of a 53,8%, similar overexpression happens in both bands.

The final differential expression of Free Ub protein after treatment with KA+Act was therefore important, since KA+Act triggered an increase of Free Ub protein expression of a 1114% and 416% for band 1 and band 2 respectively in ALS patients with respect to healthy patients.

We can observe that generally all tendencies are followed in both bands. There is a higher concentration of Free Ub in ALS cell-lines and in the case of Act and KA+Act treatments act in a different way, with an overexpression in the case of ALS and a underexpression in the case of healthy volunteers.

Expression level of UBQLN2 protein: Ubiquilin-2

Expression levels of Ubiquilin-2 (UBQLN2) quantified through WB analysis are found in *Figure 14* and *Table 14*. The graphical analysis of the averaged absolute expression levels of UBQLN2 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WBs. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments, whereas T, in this particular case, is referred to MG132.

The results show in *Figure 14* include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 87).

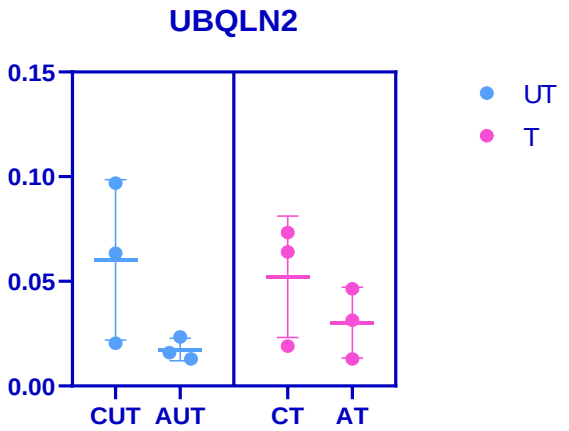


Figure 14. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of DELE1 protein.*

Table 14. *Numerical analysis of the absolute and differential UBQLN2 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	PROTEIN DEGRADATION						
Protein	Healthy	ALS	ALS vs. Healthy	Treatment	Healthy	ALS	ALS vs Healthy
UBQLN2 Anexe_WB6	6,03E-02± 3,84E-02	1,75E-02± 0,54E-02	-71,0%	MG132 (T)	5,22E-02± 2,90E-02	3,03E-02± 1,68E-02	-42,0%

As concluded from *Figure 14* and *Table 14*:

a) The values of the absolute and relative expression levels of UBQLN2 protein before treatment indicate that UBQLN2 is significantly underexpressed in ALS patients with respect to healthy patients, with an expression level of UBQLN2 of a -71,0% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of UBQLN2 protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with MG132 expression level of UBQLN2 is almost non affected in the case of Healthy volunteers, with the inhibition of expression of UBQLN2 of a -13,4%. On the contrary, expression level of UBQLN2 is significantly affected by this treatment in case of ALS patients, with the induction of expression of UBQLN2 of a 73,1%.

  The final differential expression of UBQLN2 protein after treatment with MG132 was therefore moderate, since MG132 triggered a decrease of UBQLN2 protein expression of a -42,0% in ALS patients with respect to healthy patients.

We can observe that there is significantly less UBQLN2 in ALS cells and MG132 affects significantly to ALS cells contrary to controls.

Expression level of VCP protein: Valosin-containing protein

Expression levels of Valosin-containing protein (VCP) quantified through WB analysis are found in *Figure 15* and *Table 15*. The graphical analysis of the averaged absolute expression levels of VCP protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments.

The results show in *Figure 15* left, are the values obtained after performing a single experiment with each sample (n=1). The WB analysis has been omitted in the main text, but the reader is invited to analyze them in the annexes (page 82).

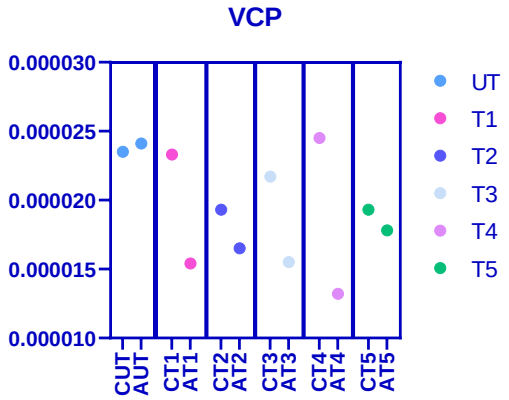


Figure 15. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of VCP protein.*

Table 15. *Numerical analysis of the absolute and differential VCP expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**PROTEIN DEGRADATION**						
Protein	**Healthy**	**ALS**		**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
VCP Anexe_WB1	2,35E-05	2,41E-05	2,55%	CLQ (T1)	2,33E-05	1,54E-05	-33,9%
				KA (T2)	1,93E-05	1,65E-05	-14,5%
				2DG (T3)	2,17E-05	1,55E-05	-28,6%
				Act (T4)	2,45E-05	1,32E-05	-46,1%
				KA+Act (T5)	1,93E-05	1,78E-05	-7,77%

As concluded from *Figure 15* and *Table 15:*

a) The values of the absolute and relative expression levels of VCP protein before treatment indicate that VCP is insignificantly overexpressed in ALS patients with respect to healthy patients, with an expression level of VCP of a 2,55% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of VCP protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of VCP is not affected in the case of Healthy volunteers. On the contrary, expression level of VCP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of VCP of a -36,1%

  The final differential expression of VCP protein after treatment with CLQ was therefore moderate, since CLQ triggered a decrease of VCP protein expression of a -33,9% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of VCP is moderately affected in the case of Healthy volunteers, with the inhibition of expression of VCP of a -68,6%. In the same line, expression level of VCP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of VCP of a -31,5%.

  The final differential expression of VCP protein after treatment with KA was therefore insignificant, since KA triggered a decrease of VCP protein expression of a -14,5% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of VCP is almost non affected in the case of Healthy volunteers, with the inhibition of expression of VCP of a -7,66%. In the same line, expression level of VCP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of VCP of a -35,7%.

  The final differential expression of VCP protein after treatment with 2DG was therefore moderate, since 2DG triggered a decrease of VCP protein expression of a -28,6% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of VCP is not affected in the case of Healthy volunteers. On the contrary, expression level of VCP is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of VCP of a -45,2%.

  The final differential expression of VCP protein after treatment with Act was therefore moderate, since Act triggered a decrease of VCP protein expression of a -46,1% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of VCP is moderately affected in the case of Healthy volunteers, with the inhibition of expression of VCP of a -60,4%. In the same line, expression level of VCP is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of VCP of a -26,1%.

  The final differential expression of VCP protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered a decrease of VCP protein expression of a -7,77% in ALS patients with respect to healthy patients.

We can observe that before treatment VCP level is almost similar in both control and patients. In the case of CLQ we cannot observe any difference in healthy volunteers, whereas we can see it in patients and same happens with Actinonin.

Expression level of LC3B protein: Autophagy-related protein

Expression levels of autophagy-related protein (LC3B) quantified through WB analysis are found in *Figure 16* and *Table 16*. The graphical analysis of the averaged absolute expression levels of LC3B protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments, whereas T, in this particular case, is referred to CLQ.

The results show in *Figure 16* left, are the values obtained after performing a single experiment with each sample (n=1), whereas the results shown in *Figure 16* right, include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 83 and 85).

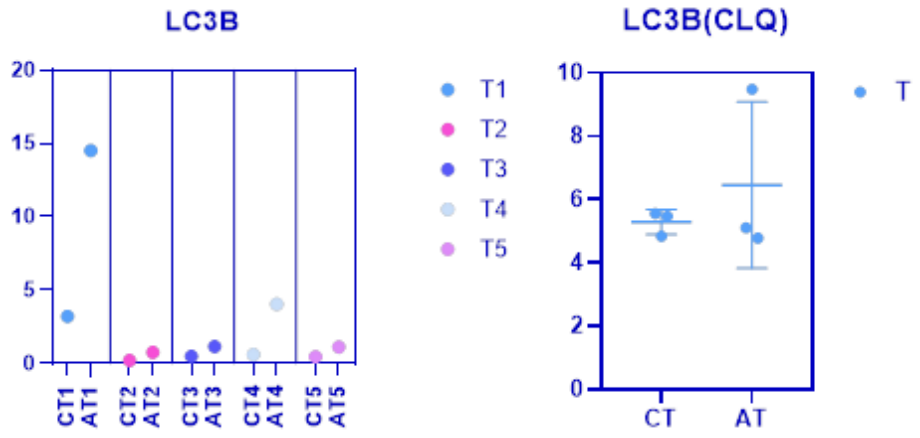


Figure 16. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of LC3B protein.*

Table 16. *Numerical analysis of the absolute and differential LC3B expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**AUTOPHAGY**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
LC3B Anexe_ WB4	0,463±0,152	0,454±0,101	-1,94%	CLQ (T)	0,829± 0,235	0,888± 0,194	7,12%
LC3B Anexe_ WB2	0,818	0,155	-81,0%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	2,71 0,257 0,118 0,656 0,575	4,11 0,155 0,154 0,402 0,188	51,7% -39,7% 30,5% -38,7% -67,3%

As concluded from *Figure 16* and *Table 16*:

a) The values of the absolute and relative expression levels of LC3B protein before treatment indicate that LC3B is moderately underexpressed in ALS patients with respect to healthy patients, with an expression level of LC3B of between -5-80% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of LC3B protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of LC3B is importantly affected in the case of Healthy volunteers, with the induction of expression of LC3B of an 80-270% In the same line, expression level of LC3B is importantly affected by this treatment in case of ALS patients, with the induction of expression of LC3B of a 90-2000%.

  The final differential expression of LC3B protein after treatment with CLQ was therefore moderate, since CLQ triggered an increase of LC3B protein expression of a 7-50% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of LC3B is moderately affected in the case of Healthy volunteers, with the inhibition of expression of LC3B of a -68,6%. On the contrary, expression level of LC3B is not affected by this treatment in case of ALS patients.

  The final differential expression of LC3B protein after treatment with KA was therefore moderate, since KA triggered a decrease of LC3B protein expression of a -39,7% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of LC3B is significantly affected in the case of Healthy volunteers, with the inhibition of expression of LC3B of a -85,6%. On the contrary, expression level of LC3B is not affected by this treatment in case of ALS patients.

  The final differential expression of LC3B protein after treatment with 2DG was therefore moderate, since 2DG triggered an increase LC3B protein expression of a 30,5% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of LC3B is almost non affected in the case of Healthy volunteers, with the inhibition of expression of LC3B of a -19,8%. On the contrary, expression level of LC3B is importantly affected by this treatment in case of ALS patients, with the induction of expression of LC3B of a 159%.

  The final differential expression of LC3B protein after treatment with Act was therefore moderate, since Act triggered a decrease of LC3B protein expression of a -38,7% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of LC3B is moderately affected in the case of Healthy volunteers, with the inhibition of expression of LC3B of a -29,7%. On the contrary, expression level of LC3B is moderately affected by this treatment in case of ALS patients, with the induction of expression of LC3B of a 21,3%.

  The final differential expression of LC3B protein after treatment with KA+Act was therefore moderate, since KA+Act triggered a decrease of LC3B protein expression of a -67,3% in ALS patients with respect to healthy patients.

We can observe that 2DG and KA do not affect ALS cell-line expression. In the case of actinonin, is seen that ALS cells are overexpressed, while controls are underexpressed.

Expression level of P62 protein: Ubiquitin binding protein

Expression levels of ubiquitin binding protein (P62), also known as SQSTM1 quantified through WB analysis are found in *Figure 17* and *Table* 17. The graphical analysis of the averaged absolute expression levels of P62 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments. The results show in *Figure 17* are the values obtained after performing a single experiment with each sample (n=1). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 82).

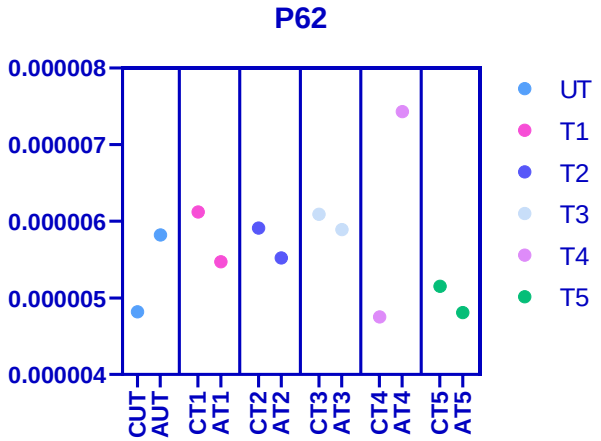


Figure 17. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of P62 protein.*

Table 17. *Numerical analysis of the absolute and differential P62 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	AUTOPHAGY						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
P62 Anexe_ WB1	4,82E-06	5,82E-06	20,7%	CLQ (T1)	6,12E-06	5,47E-06	-10,6%
				KA (T2)	5,91E-06	5,52E-06	-6,60%
				2DG (T3)	6,09E-06	5,89E-06	-3,28%
				Act (T4)	4,75E-06	7,43E-06	56,4%
				KA+Act (T5)	5,15E-06	4,81E-06	-6,60%

As concluded from *Figure 17* and *Table 17:*

a) The values of the absolute and relative expression levels of P62 protein before treatment indicate that P62 is moderately overexpressed in ALS patients with respect to healthy patients, with an expression level of P62 of between 20,7% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of P62 protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of P62 is moderately affected in the case of Healthy volunteers, with the induction of expression of P62 of a 27,0%. On the contrary, expression level of P62 is not affected by this treatment in case of ALS patients.

  The final differential expression of P62 protein after treatment with CLQ was therefore insignificant, since CLQ triggered a decrease of P62 protein expression of a -10,6% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of P62 is not affected in the case of Healthy volunteers. In the same line, expression level of P62 is not affected by this treatment in case of ALS patients.

  The final differential expression of P62 protein after treatment with KA was therefore insignificant, since KA triggered a decrease of P62 protein expression of a -6,60% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of P62 is moderately affected in the case of Healthy volunteers, with the induction of expression of P62 of a 26,3%. On the contrary, expression level of P62 is not affected by this treatment in case of ALS patients.

  The final differential expression of P62 protein after treatment with 2DG was therefore insignificant, since 2DG triggered a decrease of P62 protein expression of a -3,28% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of P62 is not affected in the case of Healthy volunteers. On the contrary, expression level of P62 is moderately affected by this treatment in case of ALS patients, with the induction of expression of P62 of a 27,7%.

The final differential expression of P62 protein after treatment with Act was therefore moderate, since Act triggered a decrease of P62 protein expression of a -38,7% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of P62 is not affected in the case of Healthy volunteers. On the contrary/In the same line, expression level of P62 is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of P62 of a -17,4%.

  The final differential expression of P62 protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered a decrease of P62 protein expression of a -6,60% in ALS patients with respect to healthy patients.

We can observe that before treatment both are quite similar and after treatment modulation in P62 expression is very moderate or even insignificant.

Expression level of CD147 protein: Cluster of differentiation 147

Expression levels of Cluster of differentiation 147 (CD147) quantified through WB analysis are found in *Figure 18* and *Table 18*. The graphical analysis of the averaged absolute expression levels of CD147 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T makes reference in this particular case to MG132.

The results show in *Figure 18* include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 85).

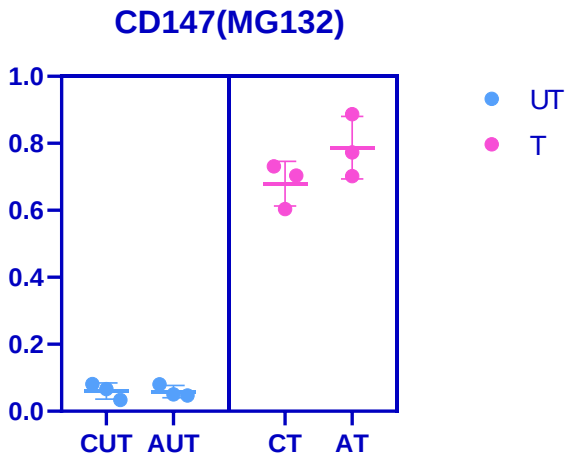


Figure 18. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of CD147 protein.*

Table 18. *Numerical analysis of the absolute and differential CD147 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**PROTEOSOME**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
CD147 Anexe_WB4	0,0603± 0,0240	0,0591± 0,0182	-1,99%	CLQ (T)	0,680± 0,067	0,760± 0,052	11,8%

As concluded from *Figure 18* and *Table 18:*

a) The values of the absolute and relative expression levels of CD147 protein before treatment indicate that CD147 is insignificantly underexpressed in ALS patients with respect to healthy patients, with an expression level of CD147 of a -1,99% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of CD147 protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of CD147 is importantly affected in the case of Healthy volunteers, with the induction of expression of CD147 of a 1028%. In the same line, expression level of CD147 is importantly affected by this treatment in case of ALS patients, with the induction of expression of CD147 of a 1186%.

  The final differential expression of CD147 protein after treatment with CLQ was therefore insignificant, since CLQ triggered an increase of CD147 protein expression of a 11,8% in ALS patients with respect to healthy patients.

We can observe that there are similar CD147 level in the cell-lines and with CLQ, is more induced in ALS.

3.3. Checking dysfunctions in glucose metabolism

Below is found the WB results obtained for key proteins implicated in the glycolysis and oxidative phosphorylation pathways. The proteins analysed are HxKI and HxKII that are related to the first process, with MTCO1 and MTCO2 that are related to OXPHOS. Those proteins are going to be observed, so as to analyse the hypothetical energetical issues ta could be in sALS.

Expression level of HxKI protein: Hexokinase I

Expression levels of Hexokinase I (HxKI) quantified through WB analysis are found in *Figure 19* and *Table 19*. The graphical analysis of the averaged absolute expression levels of HxKI protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T1, T2, T3 T4 and T5 make reference to usual treatments.

The results show in *Figure 19* left, are the values obtained after performing a single experiment with each sample (n=1). The WB analysis has been omitted in the main text, but the reader is invited to analyze them in the annexes (page 84).

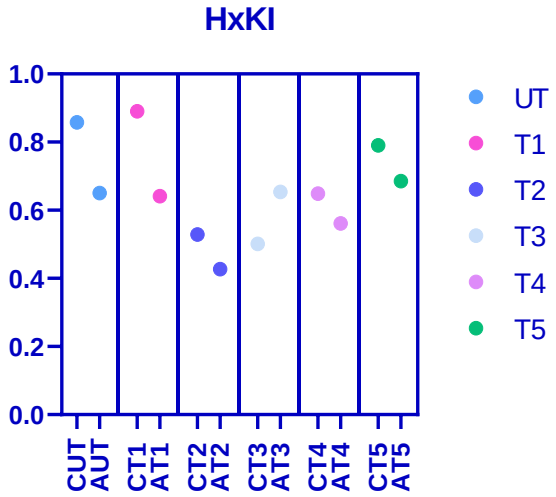


Figure 19. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of HxKI protein.*

Table 19. *Numerical analysis of the absolute and differential HxKI expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**GLYCOLYSIS**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
HxKI Anexe_WB3	0,858	0,650	-24,2	CLQ (T1)	0,890	0,541	-39,2%
				KA (T2)	0,592	0,427	-27,9%
				2DG (T3)	0,501	0,654	30,5%
				Act (T4)	0,649	0,561	-13,6%
				KA+Act (T5)	0,790	0,685	-13,3%

As concluded from *Figure 19* and *Table 19:*

a) The values of the absolute and relative expression levels of HxKI protein before treatment indicate that HxKI is moderately underexpressed in ALS patients with respect to healthy patients, with an expression level of HxKI of a -24,2% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of HxKI protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of HxKI is not affected in the case of Healthy volunteers. On the contrary, expression level of HxKI is almost non affected by this treatment in case of ALS patients, with the inhibition of expression of HxKI of a -16,8%.

  The final differential expression of HxKI protein after treatment with CLQ was therefore insignificant, since CLQ triggered a decrease of HxKI protein expression of a -39,2% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of HxKI is moderately affected in the case of Healthy volunteers, with the inhibition of expression of HxKI of a -31,0%. In the same line, expression level of HxKI is moderately affected by this treatment in case of ALS patients, with the induction/inhibition of expression of HxKI of a -34,3%.

  The final differential expression of HxKI protein after treatment with KA was therefore moderate, since KA triggered a decrease of HxKI protein expression of a -27,9% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of HxKI is moderately affected in the case of Healthy volunteers, with the inhibition of expression of HxKI of a -41,6%. On the contrary, expression level of HxKI is not affected by this treatment in case of ALS patients.

  The final differential expression of HxKI protein after treatment with 2DG was therefore moderate, since 2DG triggered an increase of HxKI protein expression of a 30,5% in ALS patients with respect to healthy patients.

- pon treatment with Act expression level of HxKI is almost non affected in the case of Healthy volunteers, with the inhibition of expression of HxKI of a -24,4%. In the same line, expression level of HxKI is insignificantly affected by this treatment in case of ALS patients, with the inhibition of expression of HxKI of a -13,7%.

  The final differential expression of HxKI protein after treatment with Act was therefore insignificant, since 2DG triggered a decrease of HxKI protein expression of a -13,6% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of HxKI is almost non affected in the case of Healthy volunteers, with the inhibition of expression of HxKI of a -7,92%. On the contrary/In the same line, expression level of Ub is insignificantly affected by this treatment in case of ALS patients, with the induction of expression of HxKI of a 5,38%.

  The final differential expression of Ub protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered a decrease of HxKI protein expression of a -13,3% in ALS patients with respect to healthy patients.

We can observe that there is less HxKI in ALS. Moreover, after treatment of 2DG in healthy volunteers is underexpressed, whereas in ALS is overexpressed.

Expression level of HxKII protein: Hexokinase II

Expression levels of Hexokinase II (HxKII) quantified through WB analysis are found in *Figure 20* and *Table 20.* The graphical analysis of the averaged absolute expression levels of HxKII protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plot T refers to CLQ in this particular case.

The results show in *Figure 20*, include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 87).

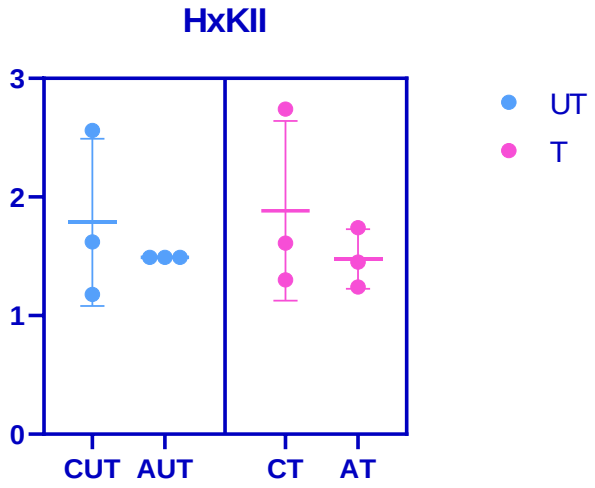


Figure 20. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of HxKII protein.*

Table 20. *Numerical analysis of the absolute and differential HxKII expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**GLYCOLYSIS**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treat-ment**	**Healthy**	**ALS**	**ALS vs Healthy**
HxKII Anexe_ WB6	1,79± 0,70	1,63± 0,14	-8,94%	CLQ (T)	1,88± 0,76	1,48±0,25	-21,3%

As concluded from *Figure 20* and *Table 20*:

a) The values of the absolute and relative expression levels of HxkII protein before treatment indicate that HxKII is insignificantly underexpressed in ALS patients with respect to healthy patients, with an expression level of HxKII of a -8,94% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of Ub protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of HxKII is not affected in the case of Healthy volunteers. In the same line, expression level of HxKI is almost non affected by this treatment in case of ALS patients, with the induction of expression of HxKII of a -9,2%.

  The final differential expression of HxKII protein after treatment with CLQ was therefore insignificant, since CLQ triggered a decrease of HxKII protein expression of a -21,3% in ALS patients with respect to healthy patients.

We can observe that there are similar concentrations of HxKII in both cell-lines and the treatment does not affect even moderately.

Expression level of MTCO1 protein: Mitochondrially Encoded Cytochrome C Oxidase I

Expression levels of Mitochondrially Encoded Cytochrome C Oxidase I (MTCO1) quantified through WB analysis are found in *Figure 21* and *Table 21.* The graphical analysis of the averaged absolute expression levels of MTCO1 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. In the plots T in this particular case, refers to MG132.

The results show in *Figure 21* include three experiments per sample (n=3), plus the average value (central horizontal line and the standard deviation value). The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 87).

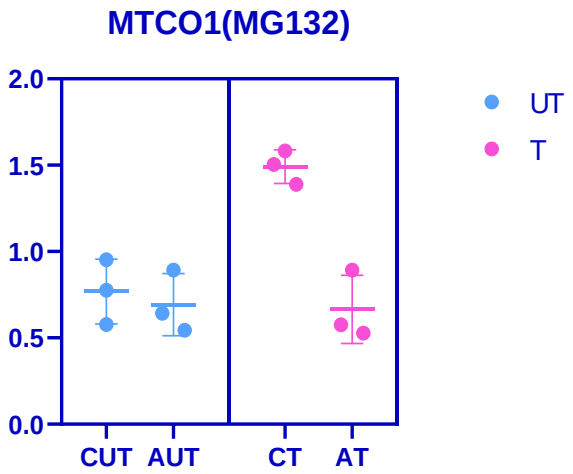


Figure 21. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of MTCO1 protein.*

Table 21. *Numerical analysis of the absolute and differential MTCO1 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**OXIDATIVE PHOSPHORYLATION**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
MTCO1 Anexe_WB6	0,769± 0,188	0,693± 0,179	-9,88%	MG132 (T)	1,49± 0,10	0,689± 0,239	-53,8%

As concluded from *Figure 21* and *Table 21:*

a) The values of the absolute and relative expression levels of MTCO1 protein before treatment indicate that MTCO1 is insignificantly underexpressed in ALS patients with respect to healthy patients, with an expression level of MTCO1 of a -9,88% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of MTCO1 protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with MG132 expression level of MTCO1 is significantly affected in the case of Healthy volunteers, with the induction of expression of MTCO1 of a 93,8%. On the contrary/In the same line, expression level of MTCO1 is not affected by this treatment in case of ALS patients.

  The final differential expression of MTCO1 protein after treatment with MG132 was therefore moderate, since MG132 triggered a decrease of MTCO1 protein expression of a -53,8% in ALS patients with respect to healthy patients.

We can observe that before treatment MTCO1 expression level is similar in both healthy volunteers and ALS patients. Apart from that, after MG132 treatment, sALS expression is maintained whereas MTCO1 is overexpressed in healthy volunteers.

Expression level of MTCO2 protein: Mitochondrially Encoded Cytochrome C Oxidase II

Expression levels of Mitochondrially Encoded Cytochrome C Oxidase II (MTCO2) quantified through WB analysis are found in *Figure 22* and *Table 22*. The graphical analysis of the averaged absolute expression levels of MTCO2 protein before and after the corresponding treatment both in healthy and ALS patients, as well as a picture of a representative WB. The WB analysis has been omitted in the main text, but the reader is invited to analyze it in the annexes (page 84 and 86).

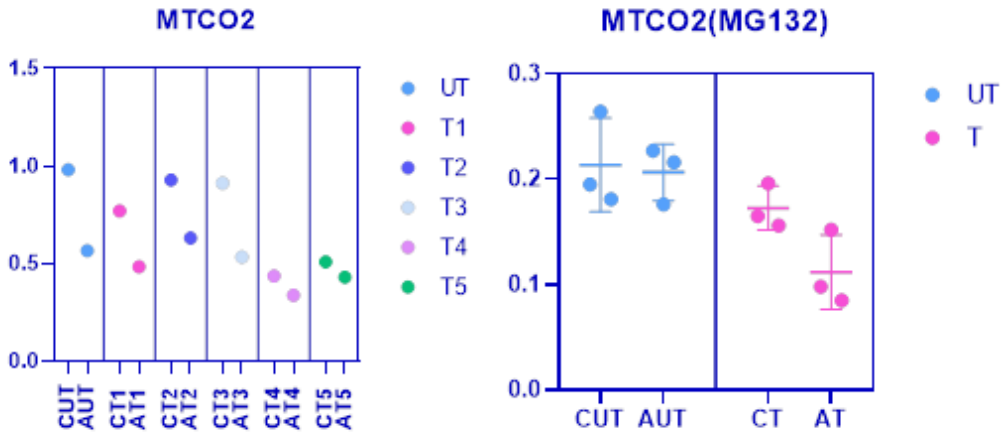


Figure 22. *Graphical representation of the data and graphical average data obtained from the quantification of absolute protein expression levels determined by WB analysis of MTCO2 protein.*

Table 22. *Numerical analysis of the absolute and differential MTCO2 expression level before and after the corresponding treatment both in healthy volunteers (Healthy) and ALS patients (ALS).*

	Before treatment			After treatment			
	Expression (a.u.) (Absolute value)		Differential expression (%)	Expression (a.u.) (Absolute value)			Differential expression (%)
Pathway	**OXIDATIVE PHOSPHORYLATION**						
Protein	**Healthy**	**ALS**	**ALS vs. Healthy**	**Treatment**	**Healthy**	**ALS**	**ALS vs Healthy**
MTCO2 Anexe_WB5	0,213± 0,044	0,206± 0,027	-3,29%	MG132 (T)	0,172± 0,021	0,112± 0,035	-34,9%
MTCO2 Anexe_WB3	0,982	0,567	-42,3%	CLQ (T1) KA (T2) 2DG (T3) Act (T4) KA+Act (T5)	0,771 0,929 0,913 0,437 0,509	0,484 0,632 0,534 0,338 0,431	-37,2% -32,0% -41,5% -22,6% -15,3

As concluded from *Figure 22* and *Table 22:*

a) The values of the absolute and relative expression levels of MTCO2 protein before treatment indicate that MTCO2 is moderately underexpressed in ALS patients with respect to healthy patients, with an expression level of MTCO2 of a -5-40% in the former with respect to the latter.

b) The values of the absolute and relative expression levels of MTCO2 protein after treatment with distinct drugs reflect the following interesting facts:

- Upon treatment with CLQ expression level of MTCO2 is almost not affected in the case of Healthy volunteers, with the inhibition of expression of MTCO2 of a -21,5%. In the same line, expression level of MTCO2 is insignificantly affected by this treatment in case of ALS patients, with the inhibition of expression of MTCO2 of a -14,6%.

  The final differential expression of MTCO2 protein after treatment with CLQ was therefore moderate, since CLQ triggered a decrease of MTCO2 protein expression of a -37,2% in ALS patients with respect to healthy patients.

- Upon treatment with KA expression level of MTCO2 is not affected in the case of Healthy volunteers. In the same line, expression level of MTCO2 is almost not affected by this treatment in case of ALS patients, with the induction of expression of MTCO2 of a 11,5.

  The final differential expression of MTCO2 protein after treatment with KA was therefore moderate, since KA triggered an increase/decrease of MTCO2 protein expression of a -32,0% in ALS patients with respect to healthy patients.

- Upon treatment with 2DG expression level of MTCO2 is not affected in the case of Healthy volunteers. In the same line, expression level of MTCO2 is not affected by this treatment in case of ALS patients.

  The final differential expression of MTCO2 protein after treatment with 2DG was therefore moderate, since 2DG triggered a decrease of MTCO2 protein expression of a -41,5% in ALS patients with respect to healthy patients.

- Upon treatment with Act expression level of MTCO2 is moderately affected in the case of Healthy volunteers, with the inhibition of expression of MTCO2 of a -55,5%. In the same line, expression level of MTCO2 is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of MTCO2 of a -40,4%.

  The final differential expression of MTCO2 protein after treatment with Act was therefore insignificant, since Act triggered a decrease of MTCO2 protein expression of a -22,6% in ALS patients with respect to healthy patients.

- Upon treatment with KA+Act expression level of MTCO2 is moderately affected in the case of Healthy volunteers, with the inhibition of expression of MTCO2 of a -48,2%. In the same line, expression level of MTCO2 is almost not affected by this treatment in case of ALS patients, with the inhibition of expression of MTCO2 of a -24,0%.

  The final differential expression of MTCO2 protein after treatment with KA+Act was therefore insignificant, since KA+Act triggered a decrease of MTCO2 protein expression of a -15,3% in ALS patients with respect to healthy patients.

- Upon treatment with MG132 expression level of MTCO2 is almost not affected in the case of Healthy volunteers, with the inhibition of expression of MTCO2 of a -19,2%. In the same line, expression level of MTCO2 is moderately affected by this treatment in case of ALS patients, with the inhibition of expression of MTCO2 of a -45,6%.

  The final differential expression of MTCO2 protein after treatment with MG132 was therefore moderate, since MG132 triggered a decrease of MTCO2 protein expression of a -34,9% in ALS patients with respect to healthy patients.

We can observe that lower concentration of MTCO2 can be found in ALS. Apart from da, MG132 inhibits more MTCO2 in the case of sALS cells. Moreover, KA and 2DG do not affect to MTCO2 expression.

Summary of the results

Table 23. *Summary of the numerical analysis of the absolute and differential protein expression level before and after treatment corresponding to ALS patients and Healthy volunteers.*

ISR	Protein expression meaning	Observations
DELE1	When the pathway is activated, DELE1 is cut. If DELE1 is overexpressed, ISR is active.	$DELE1_L$ is underexpressed in ALS; different modulators (treatments) have different effects, either increase or decrease expression. $DELE1_{SA}$ and $DELE1_{SB}$ overexpressed in ALS, and decreases with treatment.
eIF2α P	If is overexpressed, ISR is active.	Underexpressed in ALS.
CHOP	If DELE1, eIF2α P and CHOP are overexpressed, ISR is active.	Overexpressed in ALS.
BIP	If BIP is overexpressed, UPR is active.	Incongruences between experiments.
Protein degradation	**Protein expression meaning**	**Observations**
Ub	If Ub is overexpressed, there are more proteins that need to be degraded.	Incongruences between experiments, but as expected MG132 treatment has a high effect on cells and CLQ affects in a higher way to sALS cells.
Free Ub	If free Ub is overexpressed, more proteins have been degraded or there's a need for degradation.	More Free Ub in ALS cells and with Act and KA+Act, we have an overexpression, whereas is underexpressed in controls.
VCP	If is overexpressed, proteosome will be active or there will be a need for protein degradation.	Before treatment expression level is similar, but after treatment CLQ and Act ALS cell-lines are more affected.
UBQLN2	If is overexpressed, there is a need of protein degradation, proteosome will be active.	Significantly less UBQLN2 in ALS cells and with MG132 is induced in ALS cells.
CD147	If is overexpressed, proteins form ERAD must be degraded.	Similar before treatment but with CLQ is induced in ALS cells.
LC3B	If is overexpressed, means that proteins need to be degraded or that autophagosome if formed and proteins are degraded.	KA and 2DG does not affect the cells and in the case of Act, is overexpressed in ALS cells whereas is underexpressed in controls.
P62	If is overexpressed means that autophagy is active.	Insignificant differences between cell-lines.
Glycolysis	**Protein expression meaning**	**Observations**
HxKI	If is overexpressed means that the glycolysis is activated from the first step.	Less HxKI in ALS, after treatment with 2DG in underexpressed in controls but overexpressed in ALS.
HxKII	If is overexpressed means that the glycolysis is activated from the first step.	Very similar values were observed.
OXPHOS	**Protein expression meaning**	**Observations**
MTCO2	If is overexpressed, means that the fourth complex of the TCA cycle is active.	Before treatment, lower concentration in ALS and is inhibited after treatment with MG132.
MTCO1	If is overexpressed, means that the third complex of the TCA cycle is active.	Before treatment similar values, sALS is not affected by MG132 and in controls is overexpressed.

4. DISCUSSION

As it has been observed through the results, there are evident differences between healthy and patient cells. In this preliminary semiquantitative study, three main pathways that have been reported to be related with neurodegeneration aggravation and specifically with ALS (ISR, protein degradation and dysfunction in glycolysis metabolism) and they have been analysed. In the quantification done by WB technique, some significant differences have been observed and making use of the literature and all theoretical bases perused in the introduction, the discussion will be focused toward obtaining some logical conclusions as to the molecular basis of the disease respect.

The Inner stress response has been reported to be related tightly to neurodegenerative diseases, specially inside the UPR different cascades, PERK eIF2α kinase route has been related to it. However, lately research statements have mentioned that maybe the overexpression of CHOP or ATF4 does not derive from UPR but from the OMA1-DELE1-HRI pathway. So as to prove this, DELE1 and BIP proteins have been analysed. As BIP is the chaperon that activates PERK, will give us information about the situation of that pathway, whereas DELE1 describes the state of the other pathway just mentioned in the analysed cell-lines. However, the limited description of DELE1 in literature makes very difficult and challenging its interpretation.

With respect to BIP, we cannot obtain any clear result, as there are incongruences between both experiments, so it cannot be proofed if PERK is active or not. As for DELE1, is observed that in ALS cells is less $DELE1_L$, but on the contrary, $DELE1_S$ is overexpressed, which means that large part of the induction of eIF2α phosphorylation and CHOP will be produced by HRI, this is, by the heme depletion pathway. Regarding other proteins implied in the response pathway, has been observed that eIF2αP is underexpresed in ALS, whereas CHOP is overexpressed. This can seem impossible as the phosphorylation of eIF2α is what really induces ATF4 and in hence CHOP, but the thing is that phosphorylation as it is an equilibrium time-dependent reaction, we cannot obtain real data of the process or the cellular situation. Even if we cannot conclude if UPR is active or not, is observed that CHOP is overexpressed in ALS, so we can assume that ISR is active.

Concerning protein degradation, specially autophagic problems have been reported in ALS cells. Anyway, in neurons the most used degradation system is UPS specially as ERAD has seen to be active in ALS cells as a response to the stress. Ub protein overexpression should be high in ALS cells as more is needed to deal with unfolded proteins, as there is a decrease in the proteasome subunits. As for Ub some incongruences have been observed, but as Ub is part of the UPS an expected effect has happened with MG132 treatment with the induction of Ub, as proteasome cascade is stopped, an accumulation of the ubiquitinated protein that must be degraded happens. However, in most cases UT control either ALS cell have a similar Ub concentration. In the case of Free Ub there is more in ALS, so maybe more proteins need to be degraded in ALS maybe because there are more unfolded/misfolded protein and with the inhibition of cellular breathing in overexpression can be observed, whereas a contrary effect happens in controls. Anyway, the increase of Free Ub could mean several things, the cell senses that more Ub is needed in ALS cells, and they really need it because they have more damaged proteins, what should be seen with the MG132 treatment. Maybe, the process of ubiquitinating proteins is not working efficiently as in control cells and this in hence induces free Ub. A significant difference concentration of UBQLN2 is observed between cells, which play a significant role in both UPS and autophagy, so maybe this can be a critical role in sALS, which hinders the degradation of proteins. VCP protein has many functions in cellular basic mechanisms and even if VCP level is similar in both controls and patient cells, with CLQ this is, with autophagy inhibition, is moderately inhibited in ALS cells while this does not happen in control cells, anyway, is not a significant difference. Similar happens with CD147 but in the case of ALS treated with CLQ, CD147 is induced. We can see very similar values of P62 so no conclusions can be obtained from that experiment, even if after

treated with Act LC3B is significantly affected by act in ALS cells with its overexpression, contrary to control cell lines.

So as to protein degradation pathways we can say that there is a higher Free Ub concentration, that could be because of an excess of wrong folded proteins and in hence, a higher need for the degradation of those. Moreover, UBQLN2 is significantly inhibited and this protein is tightly related to neurodegeneration and specifically to ALS as is reported in many researches. So, maybe a problem in protein degradation can really be a significant cause of sALS. LC3B is overexpressed with actinonin, and this is implied in the autophagosome formation, what would affirm that a higher demand of autophagy is needed to compensate the proteasomal dysfunction Anyway, it also regulates ROS (reactive oxygen species) by preventing its excess, so this can also demonstrate that ALS cells are in a constant stress situation, at least higher than in controls.

Finally, glucose metabolism is also analysed. ALS has been related to many energetic dysfunctions and that is related to the wrong performance of glycolysis, TCA cycle or OXPHOS. In the results obtained is observed that there is less HxKI in ALS cell-lines, even if have not been seen any difference in the HxKII levels. After treatment with 2DG, an overexpression of HxKI is seen in ALS, whereas an inhibition happens in controls. This result is quite confusing, but maybe the inhibition of N-glycosylation makes a difference between both patient and healthy volunteers as the effect should be the same in both, the inhibition of HxKI. As for OXPHOS, lower concentration of MTCO2 has been observed in ALS and is inhibited by the proteasome inhibition, i.e., after treatment with MG132. In the case of MTCO1, before treatment control and patients have similar values, but after treatment with MG132 sALS cells are not affected.

5. CONCLUSIONS AND FUTURE PLANNING

To sum up, the main objectives have been completed as by this semiquantitative previous analysis some general results have been obtained that do match with some reported results in literature. Moreover, n=3 experiments have provided us to see some common tendencies between patients, even if in some cases the standard deviation is quite big, as each case has its own characteristics. Anyway, just a single technique has been carried out and that makes it difficult to draw clear and conclusive ideas. Besides, these pathways have much more proteins than the ones analysed and to carry out the research must be taken into account more proteins so as to obtain much more evidences of this previous analysis. Anyway, with the results obtain we can assume that ISR is active in ALS as CHOP is overexpressed, but not only that, DELE1s overexpression in sALS cells proofs that not only is affected or induced by UPR but the influence of other parallel pathway exists, the one is driven by DELE1 and apart from that, can be involved in the ALS disease onset. Another important fact is the underexpression of UBQLN2. This shows that there could be a problem in the protein degradation pathway, specifically in UPS as it has been seen in literature, proteasome degradation decreases and an increase of the autophagic process is observed, as it is seen in the overexpression of LC3B. So, UBQLN2 can be a major critical cause in sALS. Moreover, more Free Ub is observed and even if this can be interpreted in many different ways, seen the UBQLN2 differential expression results we can affirm that maybe the degradation process is not working in a proper way and this increases Free Ub protein concentration. Other point of view would be that, the underexpression of UBQLN2 inhibits protein degradation processes and as this creates a stress situation in the basic cellular mechanisms, that can end in an accumulation of misfolded proteins. Regarding glucose metabolism insignificant differences can be observed in general taking into account the proteins that have been analysed.

As it has been seen during all the task, ALS involves lots of complex cellular processes and any of them can be the cause of sporadic cases. Apart from that, almost every result can have other interpretation or other point of view which involves dedicated research and many evidences so as to obtain some clear conclusions.

As mentioned above, more evidences are needed so as to proof the real state of each pathway. Western blotting is an easy, cheap and rapid semiquantitative technique and is quite practical to obtain some previous analysis and evidences, even if much more proteins of each cascade should be analysed to obtain some conclusions towards these pathways, and in the same way, the implementation of that results with other techniques must be almost essential. As for the proteins analysed, BIP must be analysed again and all the other UPR cascades maybe should also quantified at least by WB, so as to see if really UPR is not responsible for sALS and more proteins as OMA1 or HRI must be quantified to be sure that heme depletion pathway can be involved in the disease. More analyses should be done to confirm that there is really less UBQLN2 and more Free Ub and try to identify its consequence in the ALS cells also find other characteristical proteins part of autophagy and UPS degradation routes as 26S, that can proof the real state of UPS. As it has been seen in the introduction, in literature, silencing experiments have also been done to see the effect of the protein in the cell and that could be a good option search for more evidences. Glucose metabolism dysfunction has been determined as a directly related cause of ALS by many researchers, but in the experiments carried out there was no evidence of it. So it would also be important to analyse more proteins of this metabolism or even do more WB of the ones quantified to obtain clearer results and state if really the disease has nothing to do with glucose metabolism.

6. BIBLIOGRAPHY

(1) Dugger, B. N.; Dickson, D. W. Pathology of Neurodegenerative Diseases. *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor Laboratory Press 2017. https://doi.org/10.1101/cshperspect.a028035.

(2) Ragagnin, A. M. G.; Shadfar, S.; Vidal, M.; Jamali, M. S.; Atkin, J. D. Motor Neuron Susceptibility in ALS/FTD. *Frontiers in Neuroscience*. Frontiers Media S.A. 2019. https://doi.org/10.3389/fnins.2019.00532.

(3) Mejzini, R.; Flynn, L. L.; Pitout, I. L.; Fletcher, S.; Wilton, S. D.; Akkari, P. A. ALS Genetics, Mechanisms, and Therapeutics: Where Are We Now? *Frontiers in Neuroscience*. Frontiers Media S.A. December 6, 2019. https://doi.org/10.3389/fnins.2019.01310.

(4) Wales, S.; C Kiernan DSc, A. M.; Cheah MBiostat, B. C.; Burrell MBBS, J.; Zoing BNurs, M. C.; Kiernan, M. C.; Vucic, S.; Cheah, B. C.; Turner, M. R.; Eisen, A.; Hardiman, O.; Burrell, J. R.; Zoing, M. C. Seminar Amyotrophic Lateral Sclerosis. *Lancet* **2011**, *377*, 942–955. https://doi.org/10.1016/S0140.

(5) Marin, B.; Boumé diene, F.; Logroscino, G.; Couratier, P.; Babron, M. C.; Leutenegger, A. L.; Copetti, M.; Preux, P. M.; Beghi, E. Variation in World Wide Incidence of Amyotrophic Lateral Sclerosis: A Meta-Analysis. *International Journal of Epidemiology* **2017**, *46* (1), 57–74. https://doi.org/10.1093/ije/dyw061.

(6) Kirby, J.; al Sultan, A.; Waller, R.; Heath, P. The Genetics of Amyotrophic Lateral Sclerosis: Current Insights. *Degenerative Neurological and Neuromuscular Disease* **2016**, 49. https://doi.org/10.2147/dnnd.s84956.

(7) Ajroud-Driss, S.; Siddique, T. Sporadic and Hereditary Amyotrophic Lateral Sclerosis (ALS). *Biochimica et Biophysica Acta - Molecular Basis of Disease*. Elsevier April 1, 2015, pp 679–684. https://doi.org/10.1016/j.bbadis.2014.08.010.

(8) Riancho, J.; Gonzalo, I.; Ruiz-Soto, M.; Berciano, J. Why Do Motor Neurons Degenerate? Actualization in the Pathogenesis of Amyotrophic Lateral Sclerosis. *Neurologia*. Spanish Society of Neurology January 1, 2019, pp 27–37. https://doi.org/10.1016/j.nrl.2015.12.001.

(9) le Gall, L.; Anakor, E.; Connolly, O.; Vijayakumar, U. G.; Duddy, W. J.; Duguez, S. Molecular and Cellular Mechanisms Affected in Als. *Journal of Personalized Medicine*. MDPI AG September 1, 2020, pp 1–34. https://doi.org/10.3390/jpm10030101.

(10) Pakos-Zebrucka, K.; Koryga, I.; Mnich, K.; Ljujic, M.; Samali, A.; Gorman, A. M. The Integrated Stress Response. *EMBO Rep* **2016**, *17* (10), 1374–1395. https://doi.org/10.15252/embr.201642195.

(11) Zhao, L.; Zhao, J.; Zhong, K.; Tong, A.; Jia, D. Targeted Protein Degradation: Mechanisms, Strategies and Application. *Signal Transduction and Targeted Therapy*. Springer Nature December 1, 2022. https://doi.org/10.1038/s41392-022-00966-4.

(12) Tefera, T. W.; Steyn, F. J.; Ngo, S. T.; Borges, K. CNS Glucose Metabolism in Amyotrophic Lateral Sclerosis: A Therapeutic Target? *Cell and Bioscience*. BioMed Central Ltd December 1, 2021. https://doi.org/10.1186/s13578-020-00511-2.

(13) Karbowski, M.; Neutzner, A. Neurodegeneration as a Consequence of Failed Mitochondrial Maintenance. *Acta Neuropathologica*. February 2012, pp 157–171. https://doi.org/10.1007/s00401-011-0921-0.

(14) Rugarli, E. I.; Langer, T. Mitochondrial Quality Control: A Matter of Life and Death for Neurons. *EMBO Journal* **2012**, *31* (6), 1336–1349. https://doi.org/10.1038/emboj.2012.38.

(15) Tang, B. L. Glucose, Glycolysis, and Neurodegenerative Diseases. *Journal of Cellular Physiology*. Wiley-Liss Inc. November 1, 2020, pp 7653–7662. https://doi.org/10.1002/jcp.29682.

(16) Bugallo, R.; Marlin, E.; Baltanás, A.; Toledo, E.; Ferrero, R.; Vinueza-Gavilanes, R.; Larrea, L.; Arrasate, M.; Aragón, T. Fine Tuning of the Unfolded Protein Response by ISRIB Improves Neuronal Survival in a Model of Amyotrophic Lateral Sclerosis. *Cell Death and Disease* **2020**, *11* (5). https://doi.org/10.1038/s41419-020-2601-2.

(17) Wang, L.; Popko, B.; Roos, R. P. The Unfolded Protein Response in Familial Amyotrophic Lateral Sclerosis. *Human Molecular Genetics* **2011**, *20* (5), 1008–1015. https://doi.org/10.1093/hmg/ddq546.

(18) Dzhashiashvili, Y.; Monckton, C. P.; Shah, H. S.; Kunjamma, R. B.; Popko, B. The UPR-PERK Pathway Is Not a Promising Therapeutic Target for Mutant SOD1-Induced ALS. *Neurobiology of Disease* **2019**, *127*, 527–544. https://doi.org/10.1016/j.nbd.2019.03.024.

(19) Guo, X.; Aviles, G.; Liu, Y.; Tian, R.; Unger, B. A.; Lin, Y. H. T.; Wiita, A. P.; Xu, K.; Correia, M. A.; Kampmann, M. Mitochondrial Stress Is Relayed to the Cytosol by an OMA1–DELE1–HRI Pathway. *Nature* **2020**, *579* (7799), 427–432. https://doi.org/10.1038/s41586-020-2078-2.

(20) Ron, D. Translational Control in the Endoplasmic Reticulum Stress Response. *Journal of Clinical Investigation* **2002**, *110* (10), 1383–1388. https://doi.org/10.1172/jci200216784.

(21) Lu, P. D.; Harding, H. P.; Ron, D. Translation Reinitiation at Alternative Open Reading Frames Regulates Gene Expression in an Integrated Stress Response. *Journal of Cell Biology* **2004**, *167* (1), 27–33. https://doi.org/10.1083/jcb.200408003.

(22) Novoa, I.; Zeng, H.; Harding, H. P.; Ron, D.; Neibert, D. W.; Hollander, M. C.; Luethy, J. D.; Papathanasiou, M.; Fragoli, J.; Holbrook, N. J.; Hoffman-Lieberman, B. *Feedback Inhibition of the Unfolded Protein Response by GADD34-Mediated Dephosphorylation of EIF2*; 2001; Vol. 153.

(23) Zhang, S. X.; Ma, J. H.; Bhatta, M.; Fliesler, S. J.; Wang, J. J. The Unfolded Protein Response in Retinal Vascular Diseases: Implications and Therapeutic Potential beyond Protein Folding. *Progress in Retinal and Eye Research*. Elsevier Ltd March 1, 2015, pp 111–131. https://doi.org/10.1016/j.preteyeres.2014.12.001.

(24) Kim, I.; Xu, W.; Reed, J. C. Cell Death and Endoplasmic Reticulum Stress: Disease Relevance and Therapeutic Opportunities. *Nature Reviews Drug Discovery* **2008**, *7* (12), 1013–1030. https://doi.org/10.1038/nrd2755.

(25) Shacham, T.; Patel, C.; Lederkremer, G. Z. Perk Pathway and Neurodegenerative Disease: To Inhibit or to Activate? *Biomolecules*. MDPI AG March 1, 2021, pp 1–21. https://doi.org/10.3390/biom11030354.

(26) Doyle, K. M.; Kennedy, D.; Gorman, A. M.; Gupta, S.; Healy, S. J. M.; Samali, A. Unfolded Proteins and Endoplasmic Reticulum Stress in Neurodegenerative Disorders. *Journal of Cellular and Molecular Medicine*. October 2011, pp 2025–2039. https://doi.org/10.1111/j.1582-4934.2011.01374.x.

(27) Hetz, C.; Saxena, S. ER Stress and the Unfolded Protein Response in Neurodegeneration. *Nature Reviews Neurology*. Nature Publishing Group August 1, 2017, pp 477–491. https://doi.org/10.1038/nrneurol.2017.99.

(28) Bertolotti, A.; Zhang, Y.; Hendershot, L. M.; Harding, H. P.; Ron, D. *Dynamic Interaction of BiP and ER Stress Transducers in the Unfolded-Protein Response*; 2000; Vol. 2.

(29) Bartoszewska, S.; Collawn, J. F. Unfolded Protein Response (UPR) Integrated Signaling Networks Determine Cell Fate during Hypoxia. *Cellular and Molecular Biology Letters*. BioMed Central Ltd. March 13, 2020. https://doi.org/10.1186/s11658-020-00212-1.

(30) Novoa, I.; Zeng, H.; Harding, H. P.; Ron, D.; Neibert, D. W.; Hollander, M. C.; Luethy, J. D.; Papathanasiou, M.; Fragoli, J.; Holbrook, N. J.; Hoffman-Lieberman, B. *Feedback Inhibition of the Unfolded Protein Response by GADD34-Mediated Dephosphorylation of EIF2*; 2001; Vol. 153.

(31) Jousse, C.; Oyadomari, S.; Novoa, I.; Lu, P.; Zhang, Y.; Harding, H. P.; Ron, D. Inhibition of a Constitutive Translation Initiation Factor 2α Phosphatase, CReP, Promotes Survival of Stressed Cells. *Journal of Cell Biology* **2003**, *163* (4), 767–775. https://doi.org/10.1083/jcb.200308075.

(32) Taniuchi, S.; Miyake, M.; Tsugawa, K.; Oyadomari, M.; Oyadomari, S. Integrated Stress Response of Vertebrates Is Regulated by Four EIF2α Kinases. *Scientific Reports* **2016**, *6*. https://doi.org/10.1038/srep32886.

(33) Lu, P. D.; Harding, H. P.; Ron, D. Translation Reinitiation at Alternative Open Reading Frames Regulates Gene Expression in an Integrated Stress Response. *Journal of Cell Biology* **2004**, *167* (1), 27–33. https://doi.org/10.1083/jcb.200408003.

(34) Janssens, S.; Pulendran, B.; Lambrecht, B. N. Emerging Functions of the Unfolded Protein Response in Immunity. *Nature Immunology*. Nature Publishing Group January 1, 2014, pp 910–919. https://doi.org/10.1038/ni.2991.

(35) Walter, P.; Ron, D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. *Science*. American Association for the Advancement of Science November 25, 2011, pp 1081–1086. https://doi.org/10.1126/science.1209038.

(36) Fessler, E.; Eckl, E. M.; Schmitt, S.; Mancilla, I. A.; Meyer-Bender, M. F.; Hanf, M.; Philippou-Massier, J.; Krebs, S.; Zischka, H.; Jae, L. T. A Pathway Coordinated by DELE1 Relays Mitochondrial Stress to the Cytosol. *Nature* **2020**, *579* (7799), 433–437. https://doi.org/10.1038/s41586-020-2076-4.

(37) Fessler, E.; Krumwiede, L.; Jae, L. T. DELE1 Tracks Perturbed Protein Import and Processing in Human Mitochondria. *Nature Communications* **2022**, *13* (1). https://doi.org/10.1038/s41467-022-29479-y.

(38) Bradford P. Tremblay & Cole M. Haynes. Mitochondrial Distress. *Nature* **2020**, *579*, 348–349.

(39) Jane Chen; Irving M. London. Regulation of Protein Synthesis by Heme-Regulated EIF-2α Kinase. *Trends in Biochemical Sciences* **1995**, *TIBS 20*, 105–108.

(40) Donnelly, N.; Gorman, A. M.; Gupta, S.; Samali, A. The EIF2α Kinases: Their Structures and Functions. *Cellular and Molecular Life Sciences*. October 2013, pp 3493–3511. https://doi.org/10.1007/s00018-012-1252-6.

(41) Zarei, M.; Barroso, E.; Leiva, R.; Barniol-Xicota, M.; Pujol, E.; Escolano, C.; Vázquez, S.; Palomer, X.; Pardo, V.; González-Rodríguez, Á.; Valverde, Á. M.; Quesada-López, T.; Villarroya, F.; Wahli, W.; Vázquez-Carrera, M. Heme-Regulated EIF2α Kinase Modulates Hepatic FGF21 and Is Activated by PPARβ/δ Deficiency. *Diabetes* **2016**, *65* (10), 3185–3199. https://doi.org/10.2337/db16-0155.

(42) Storey, G. P.; Opitz-Araya, X.; Barria, A. Molecular Determinants Controlling NMDA Receptor Synaptic Incorporation. *Journal of Neuroscience* **2011**, *31* (17), 6311–6316. https://doi.org/10.1523/JNEUROSCI.5553-10.2011.

(43) Tai, H. C.; Schuman, E. M. Ubiquitin, the Proteasome and Protein Degradation in Neuronal Function and Dysfunction. *Nature Reviews Neuroscience*. November 2008, pp 826–838. https://doi.org/10.1038/nrn2499.

(44) Maurel, C.; Dangoumau, A.; Marouillat, S.; Brulard, C.; Chami, A.; Hergesheimer, R.; Corcia, P.; Blasco, H.; Andres, C. R.; Vourc'h, P. Causative Genes in Amyotrophic Lateral Sclerosis and Protein Degradation Pathways: A Link to Neurodegeneration. *Molecular Neurobiology*. Humana Press Inc. August 1, 2018, pp 6480–6499. https://doi.org/10.1007/s12035-017-0856-0.

(45) Badadani, M. Autophagy Mechanism, Regulation, Functions, and Disorders. *ISRN Cell Biology* **2012**, *2012*, 1–11. https://doi.org/10.5402/2012/927064.

(46) Mandhair, H. K.; Arambasic, M.; Novak, U.; Radpour, R. Molecular Modulation of Autophagy: New Venture to Target Resistant Cancer Stem Cells. *World Journal of Stem Cells* **2020**, *12* (5), 303–322. https://doi.org/10.4252/wjsc.v12.i5.303.

(47) Mariño, G.; Niso-Santano, M.; Baehrecke, E. H.; Kroemer, G. Self-Consumption: The Interplay of Autophagy and Apoptosis. *Nature Reviews Molecular Cell Biology*. February 2014, pp 81–94. https://doi.org/10.1038/nrm3735.

(48) Tefera, T. W.; Steyn, F. J.; Ngo, S. T.; Borges, K. CNS Glucose Metabolism in Amyotrophic Lateral Sclerosis: A Therapeutic Target? *Cell and Bioscience*. BioMed Central Ltd December 1, 2021. https://doi.org/10.1186/s13578-020-00511-2.

(49) Yetkin-Arik, B.; Vogels, I. M. C.; Neyazi, N.; van Duinen, V.; Houtkooper, R. H.; van Noorden, C. J. F.; Klaassen, I.; Schlingemann, R. O. Endothelial Tip Cells in Vitro Are Less Glycolytic and Have a More Flexible Response to Metabolic Stress than Non-Tip Cells. *Scientific Reports* **2019**, *9* (1). https://doi.org/10.1038/s41598-019-46503-2.

(50) Steyn, F. J.; Ioannides, Z. A.; van Eijk, R. P. A.; Heggie, S.; Thorpe, K. A.; Ceslis, A.; Heshmat, S.; Henders, A. K.; Wray, N. R.; van den Berg, L. H.; Henderson, R. D.; McCombe, P. A.; Ngo, S. T. Hypermetabolism in ALS Is Associated with Greater Functional Decline and Shorter Survival. *Journal of Neurology, Neurosurgery and Psychiatry* **2018**, *89* (10), 1016–1023. https://doi.org/10.1136/jnnp-2017-317887.

(51) Bravo, R.; Parra, V.; Gatica, D.; Rodriguez, A. E.; Torrealba, N.; Paredes, F.; Wang, Z. v.; Zorzano, A.; Hill, J. A.; Jaimovich, E.; Quest, A. F. G.; Lavandero, S. Endoplasmic Reticulum and the Unfolded Protein Response. Dynamics and Metabolic Integration. In *International Review of Cell and Molecular Biology*; Elsevier Inc., 2013; Vol. 301, pp 215–290. https://doi.org/10.1016/B978-0-12-407704-1.00005-1.

(52) Liu, M. L.; Zang, T.; Zou, Y.; Chang, J. C.; Gibson, J. R.; Huber, K. M.; Zhang, C. L. Small Molecules Enable Neurogenin 2 to Efficiently Convert Human Fibroblasts into Cholinergic Neurons. *Nature Communications* **2013**, *4*. https://doi.org/10.1038/ncomms3183.

(53) Abdullah, A. I.; Pollock, A.; Sun, T. The Path from Skin to Brain: Generation of Functional Neurons from Fibroblasts. *Molecular Neurobiology* **2012**, *45* (3), 586–595. https://doi.org/10.1007/s12035-012-8277-6.

(54) Mauthe, M.; Orhon, I.; Rocchi, C.; Zhou, X.; Luhr, M.; Hijlkema, K. J.; Coppes, R. P.; Engedal, N.; Mari, M.; Reggiori, F. Chloroquine Inhibits Autophagic Flux by Decreasing Autophagosome-Lysosome Fusion. *Autophagy* **2018**, *14* (8), 1435–1455. https://doi.org/10.1080/15548627.2018.1474314.

(55) Guo, N.; Peng, Z. MG132, a Proteasome Inhibitor, Induces Apoptosis in Tumor Cells. *Asia-Pacific Journal of Clinical Oncology*. March 2013, pp 6–11. https://doi.org/10.1111/j.1743-7563.2012.01535.x.

(56) Pajak, B.; Siwiak, E.; Sołtyka, M.; Priebe, A.; Zieliński, R.; Fokt, I.; Ziemniak, M.; Jaśkiewicz, A.; Borowski, R.; Domoradzki, T.; Priebe, W. 2-Deoxy-D-Glucose and Its Analogs: From Diagnostic to Therapeutic Agents. *International Journal of Molecular Sciences*. MDPI AG January 1, 2020. https://doi.org/10.3390/ijms21010234.

(57) Berthe, A.; Zaffino, M.; Muller, C.; Foulquier, F.; Houdou, M.; Schulz, C.; Bost, F.; de Fay, E.; Mazerbourg, S.; Flament, S. Protein N-Glycosylation Alteration and Glycolysis Inhibition Both Contribute to the Antiproliferative Action of 2-Deoxyglucose in Breast Cancer Cells. *Breast Cancer Research and Treatment* **2018**, *171* (3), 581–591. https://doi.org/10.1007/s10549-018-4874-z.

(58) Jing, C.; Li, Y.; Gao, Z.; Wang, R. Antitumor Activity of Koningic Acid in Thyroid Cancer by Inhibiting Cellular Glycolysis. *Endocrine* **2022**, *75* (1), 169–177. https://doi.org/10.1007/s12020-021-02822-x.

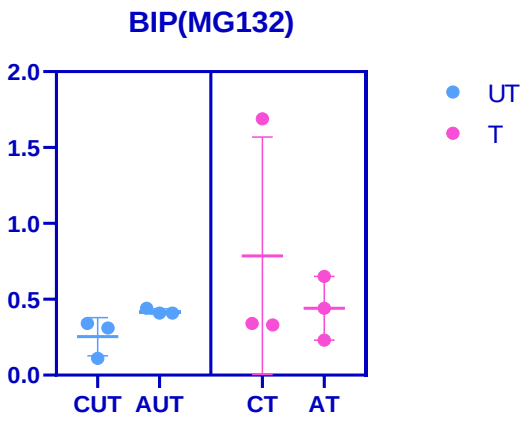
(59) Richter, U.; Lahtinen, T.; Marttinen, P.; Myöhänen, M.; Greco, D.; Cannino, G.; Jacobs, H. T.; Lietzén, N.; Nyman, T. A.; Battersby, B. J. A Mitochondrial Ribosomal and RNA Decay Pathway Blocks Cell Proliferation. *Current Biology* **2013**, *23* (6), 535–541. https://doi.org/10.1016/j.cub.2013.02.019.

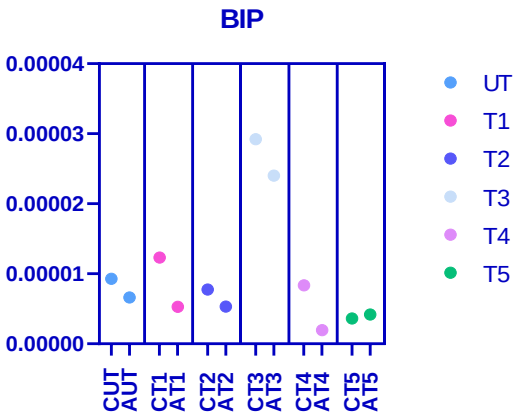
(60) Richter, U.; Lahtinen, T.; Marttinen, P.; Suomi, F.; Battersby, B. J. Quality Control of Mitochondrial Protein Synthesis Is Required for Membrane Integrity and Cell Fitness. *Journal of Cell Biology* **2015**, *211* (2), 373–389. https://doi.org/10.1083/jcb.201504062.

7. ANEXES

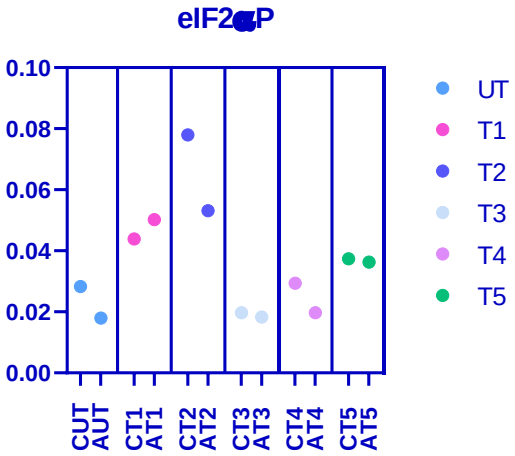
8.1. Additional plots for ISR pathway

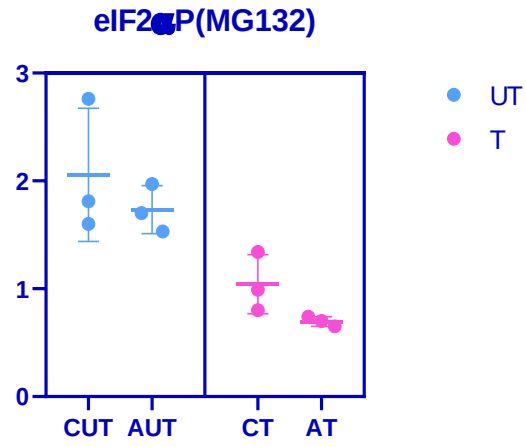
BIP

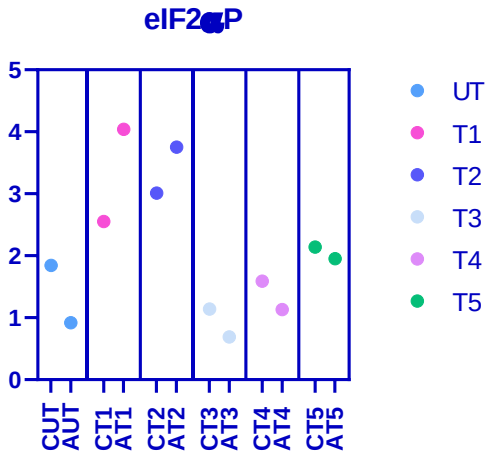




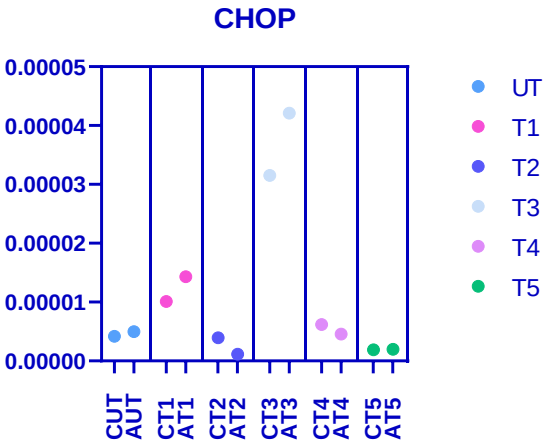
eIF2αP

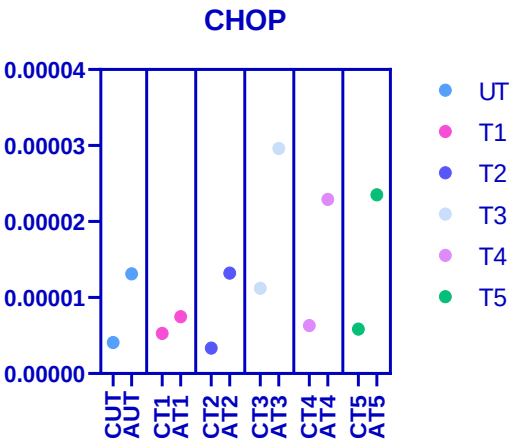


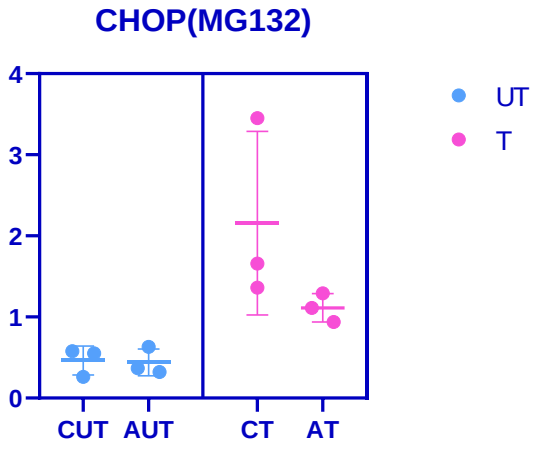


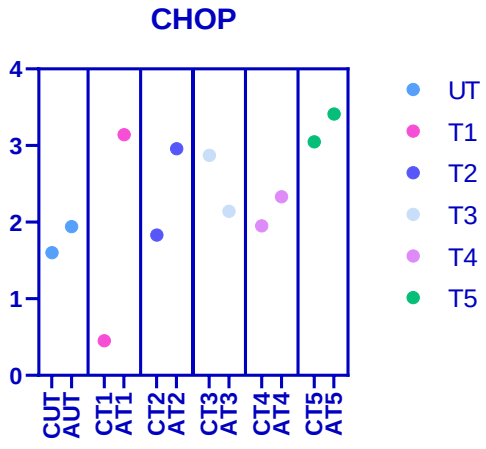

eIF2αP
5
4
3
2
1
0
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5

CHOP


CHOP
0.00005
0.00004
0.00003
0.00002
0.00001
0.00000
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5

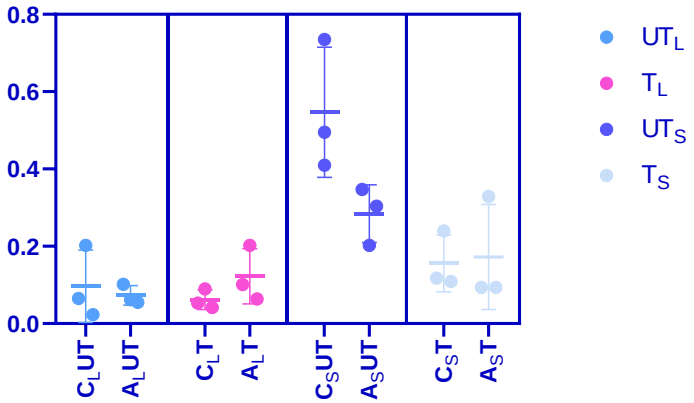

CHOP
0.00004
0.00003
0.00002
0.00001
0.00000
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5


CHOP(MG132)
4
3
2
1
0
CUT AUT
CT AT
UT
T

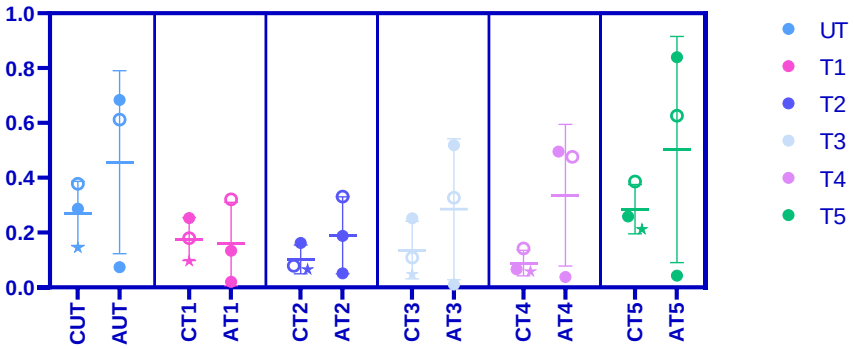

CHOP
4
3
2
1
0
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5

DELE1

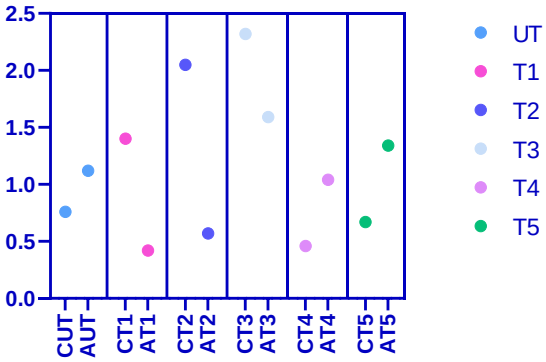
DELE1(MG132)



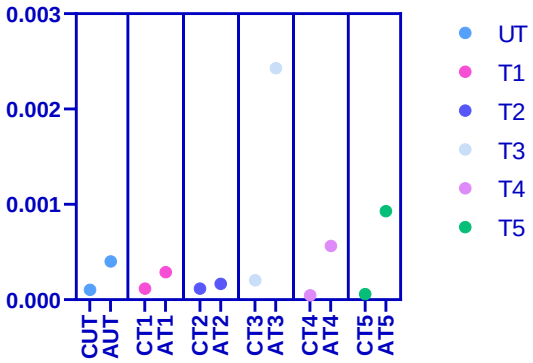
DELE1

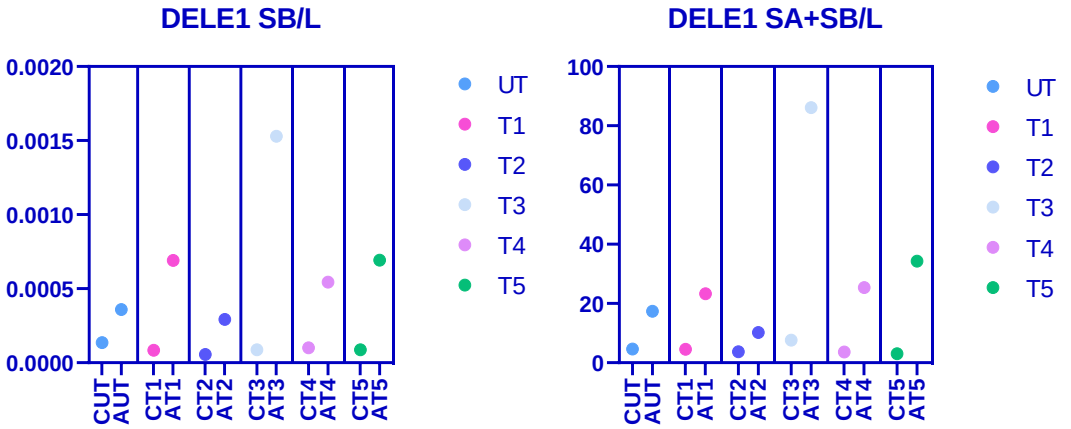


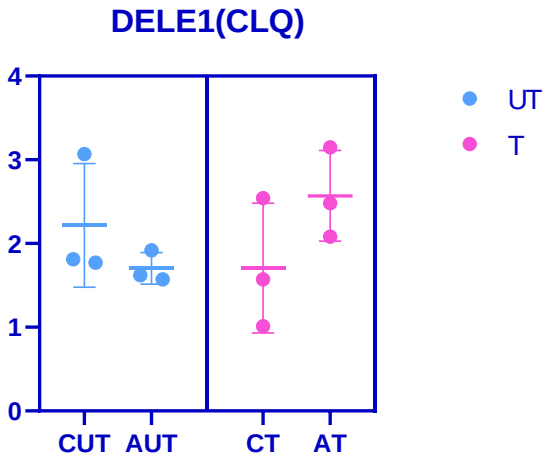
DELE1 SA/SB



DELE1 SA/L

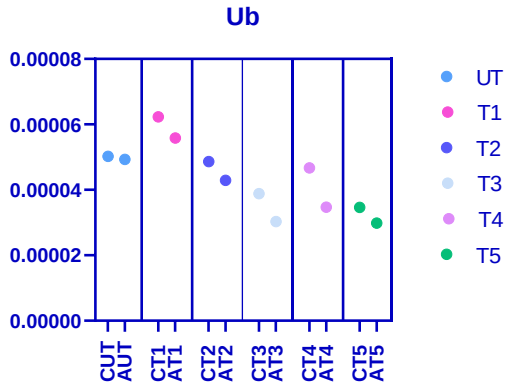


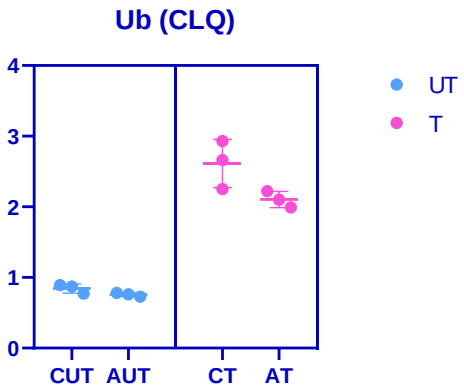

DELE1 SB/L
0.0020
0.0015
0.0010
0.0005
0.0000
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5
DELE1 SA+SB/L
100
80
60
40
20
0
CUT AUT
CT1 AT1
CT2 AT2
CT3 AT3
CT4 AT4
CT5 AT5
UT
T1
T2
T3
T4
T5

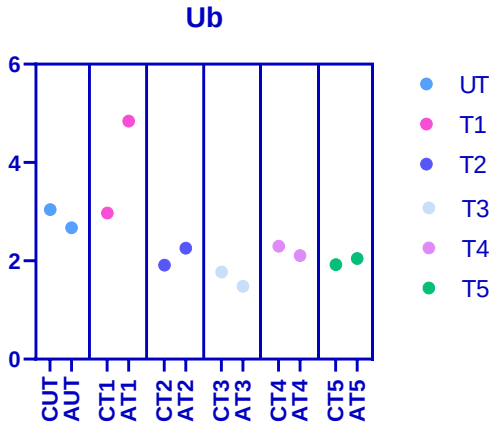

DELE1(CLQ)
4
3
2
1
0
CUT AUT
CT AT
UT
T

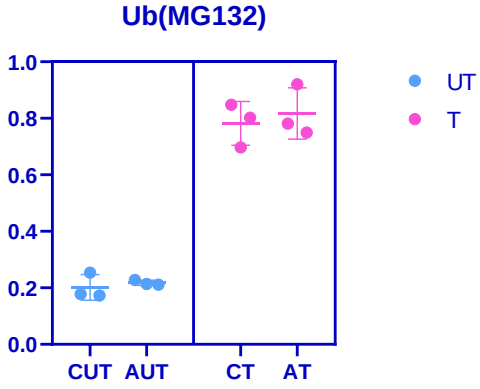
8.2. Protein degradation:

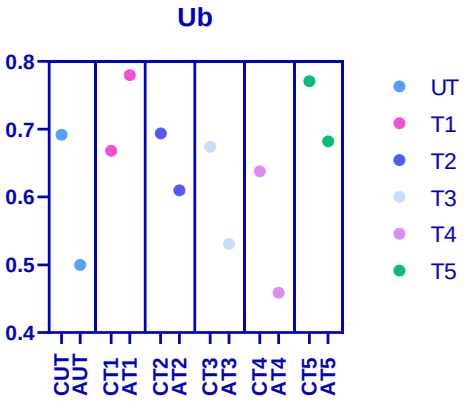
Ub

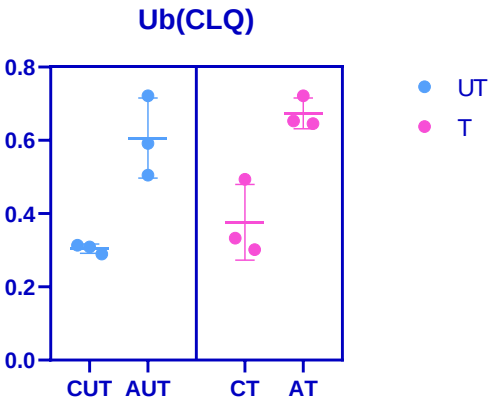


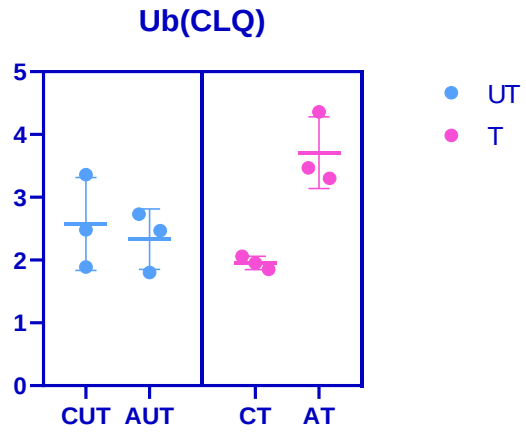




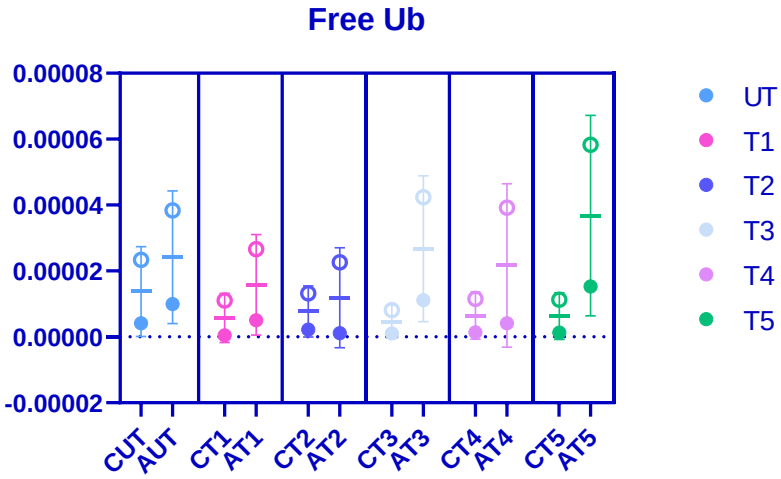




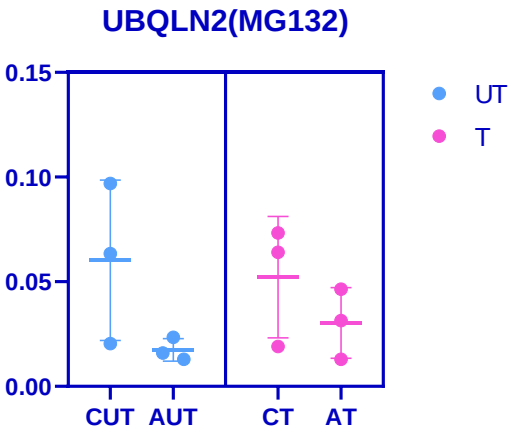




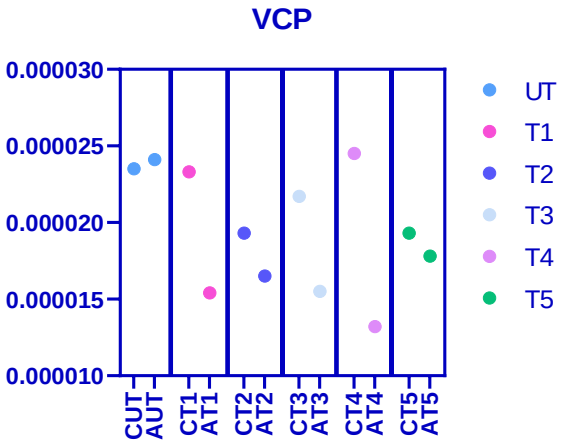
Free Ub



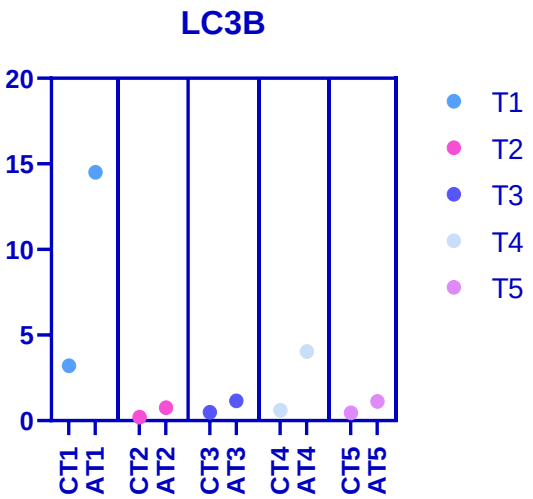
UBQLN2

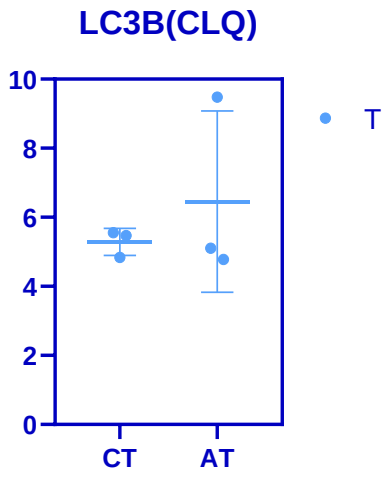


VCP

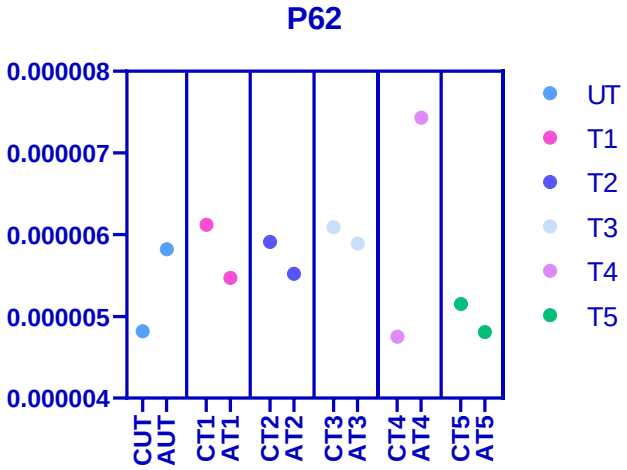

VCP
0.000030
0.000025
0.000020
0.000015
0.000010
CUT
AUT
CT1
AT1
CT2
AT2
CT3
AT3
CT4
AT4
CT5
AT5
UT
T1
T2
T3
T4
T5

LC3B

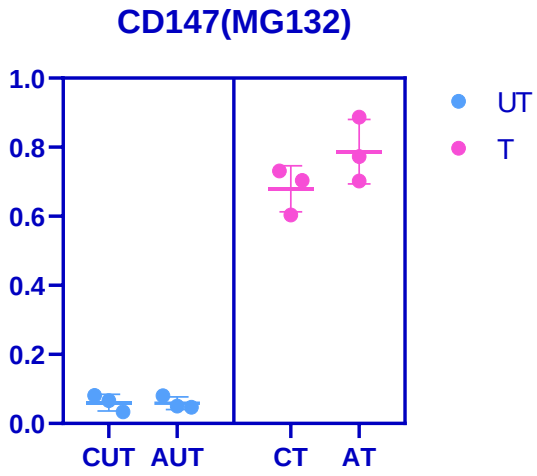

LC3B
20
15
10
5
0
CT1
AT1
CT2
AT2
CT3
AT3
CT4
AT4
CT5
AT5
T1
T2
T3
T4
T5


LC3B(CLQ)
10
8
6
4
2
0
CT
AT
T

P62

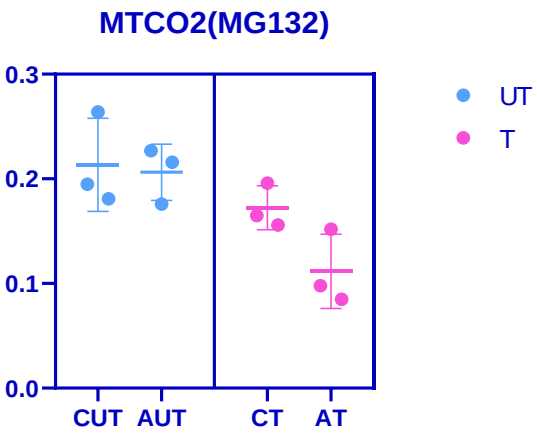

P62
0.000008
0.000007
0.000006
0.000005
0.000004
CUT
AUT
CT1
AT1
CT2
AT2
CT3
AT3
CT4
AT4
CT5
AT5
UT
T1
T2
T3
T4
T5

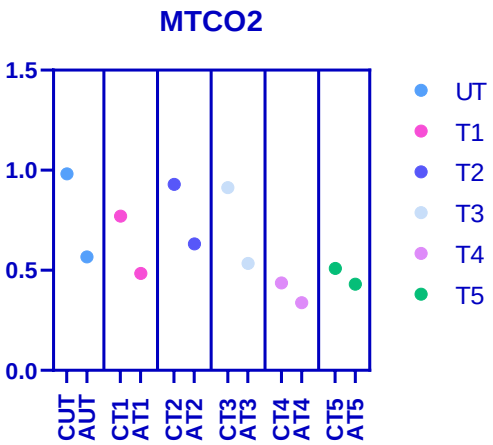
CD147


CD147(MG132)
1.0
0.8
0.6
0.4
0.2
0.0
CUT
AUT
CT
AT
UT
T

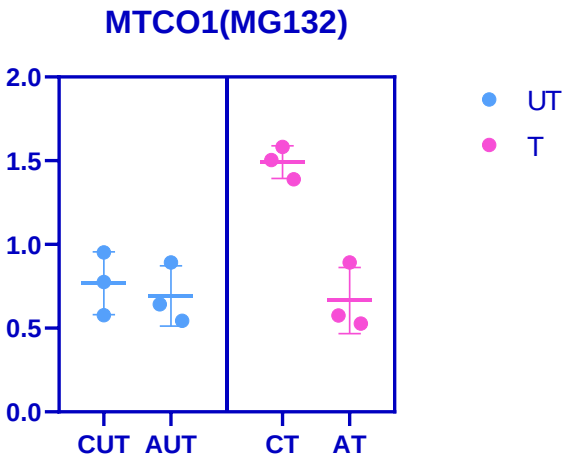
8.3. Dysfunction in glucose metabolism:

MTCO2

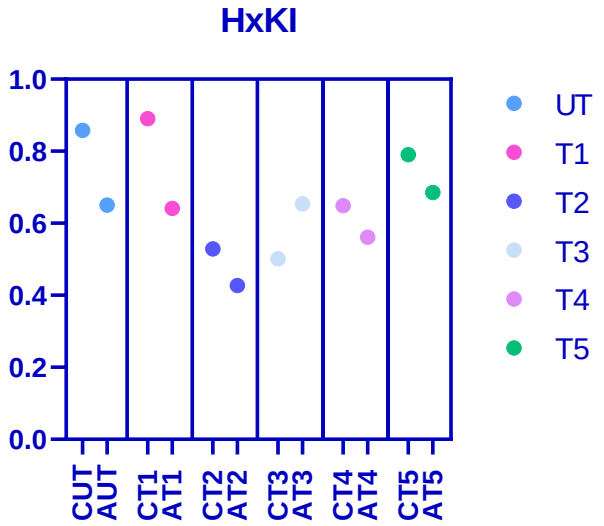




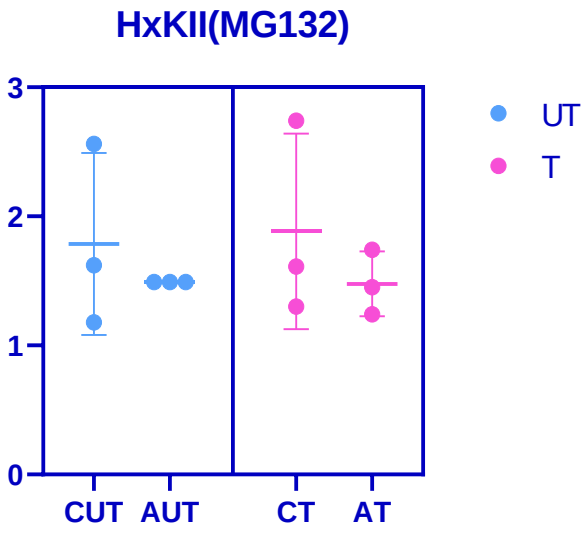
MTCO1



HxKI

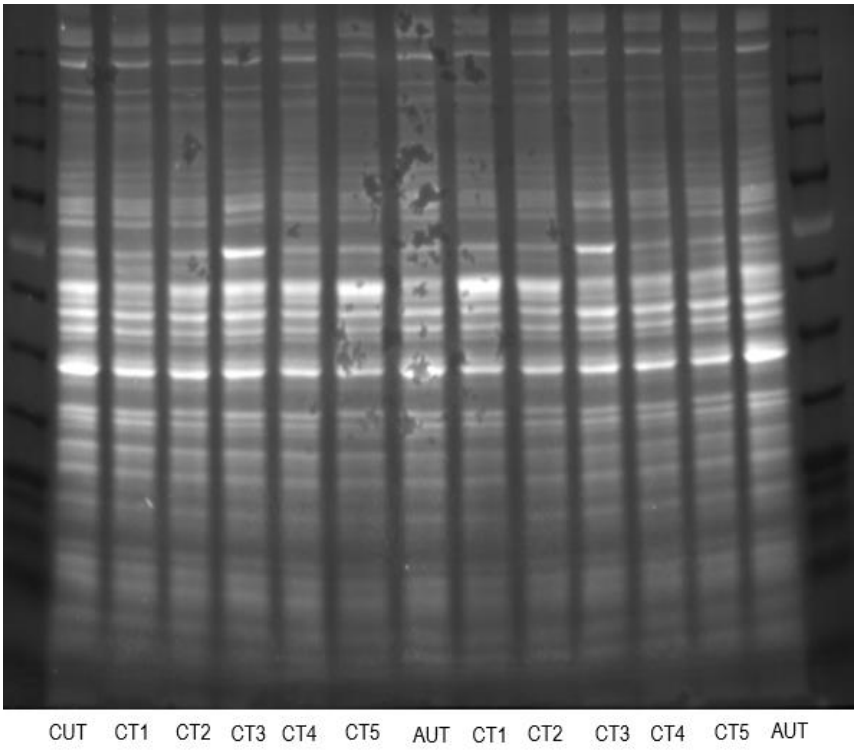

HxKI
1.0
0.8
0.6
0.4
0.2
0.0
CUT
AUT
CT1
AT1
CT2
AT2
CT3
AT3
CT4
AT4
CT5
AT5
UT
T1
T2
T3
T4
T5

HxKII

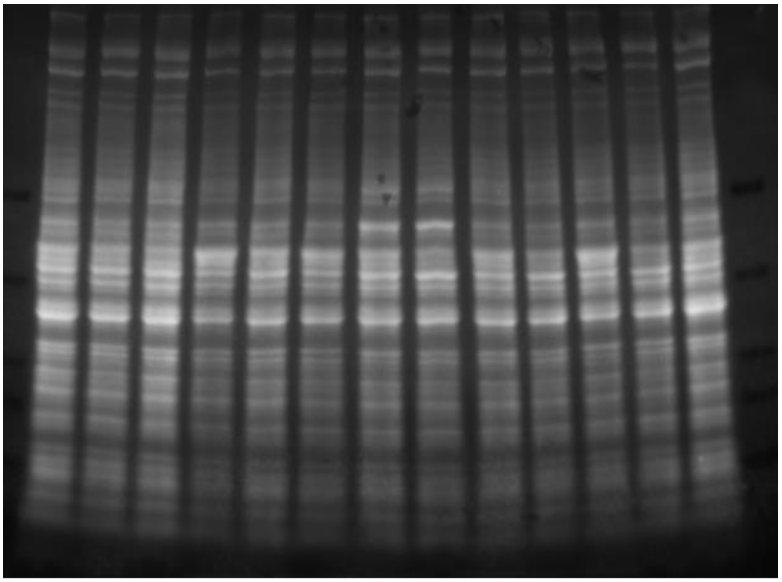

HxKII(MG132)
3
2
1
0
CUT
AUT
CT
AT
UT
T

8.4. Additional WBs

Anexe_WB1



Anexe_WB2

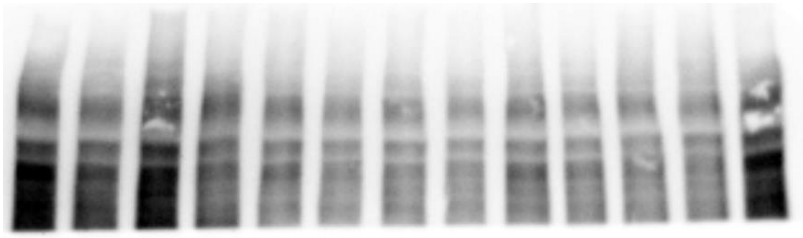


CHOP:

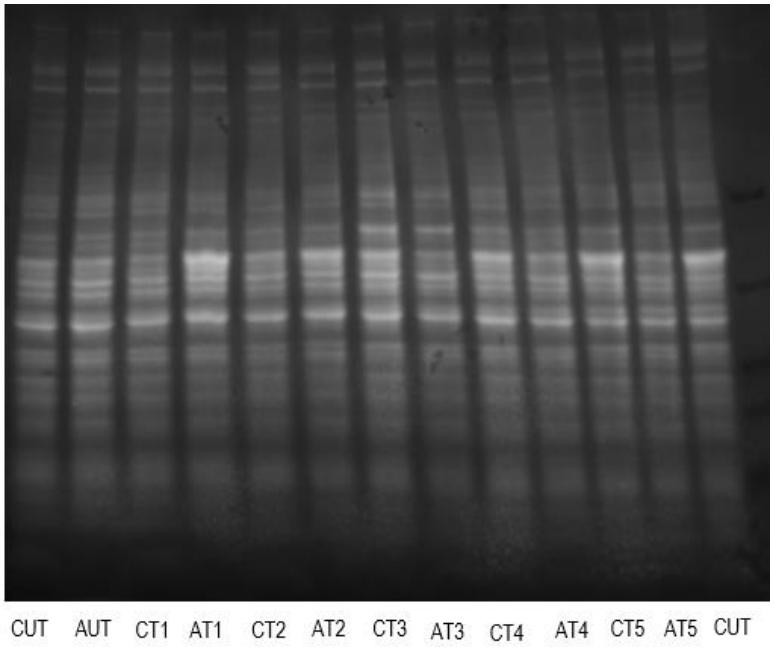
eIF2αP:

LC3B:

Ub:



Anexe_WB3

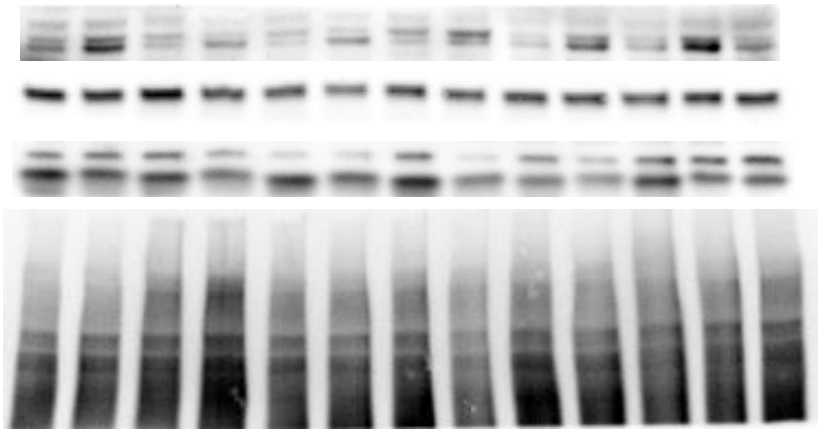


DELE1:

HxKI:

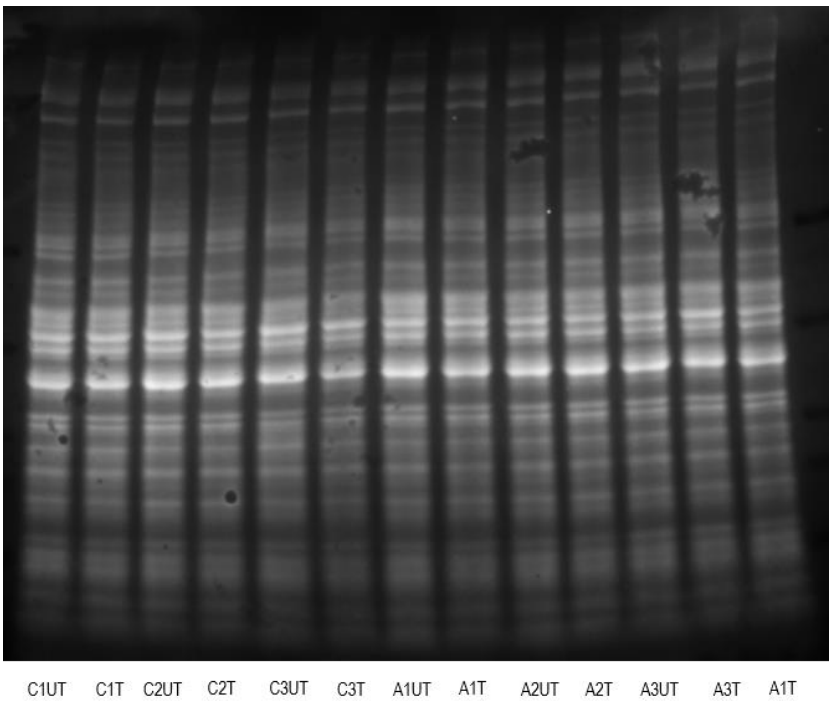
MTCO2:

Ub:



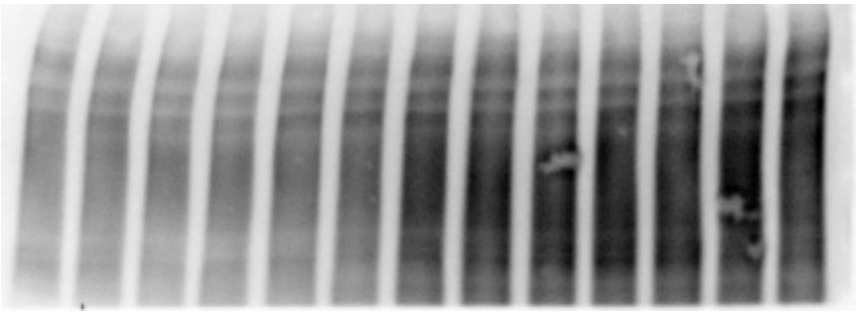
Anexe_WB4

CLQ



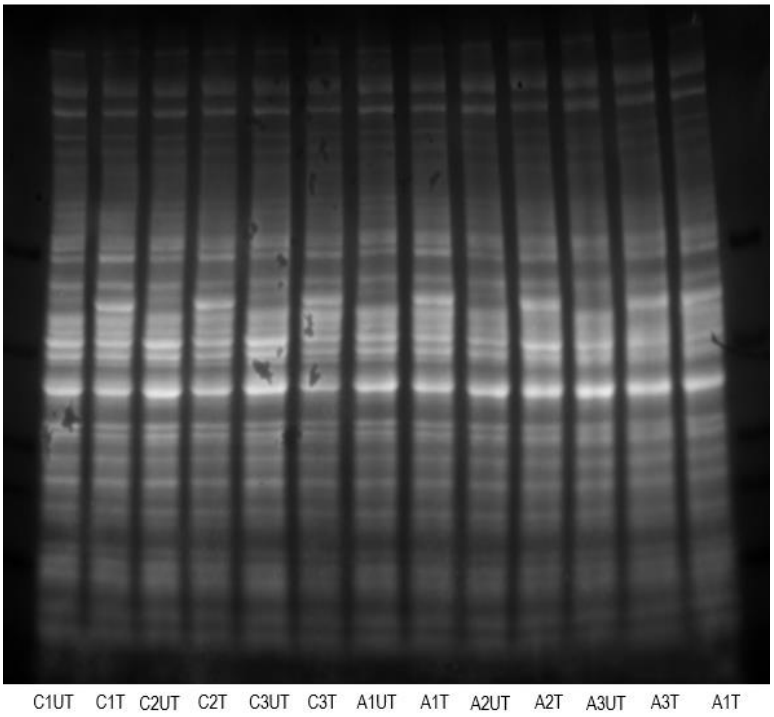
LC3B/A:

Ub:



Anexe_WB5

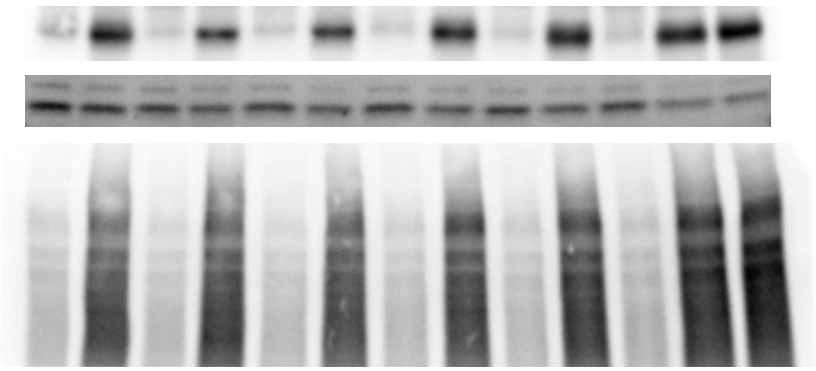
MG132



CD147:

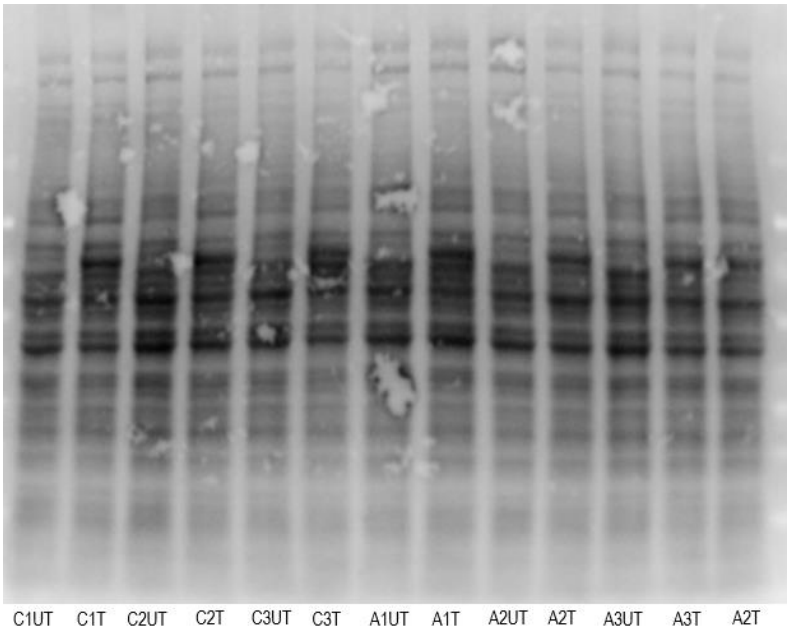
MTCO2:

Ub:



Anexe_WB6

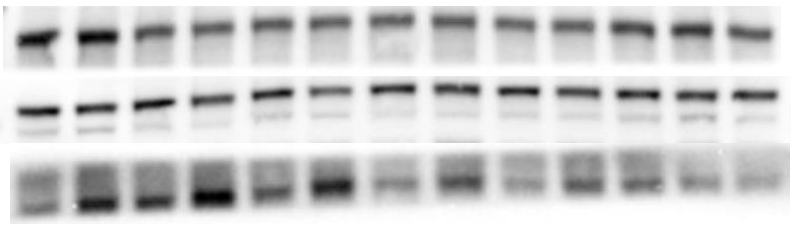
MG132



HxKII:

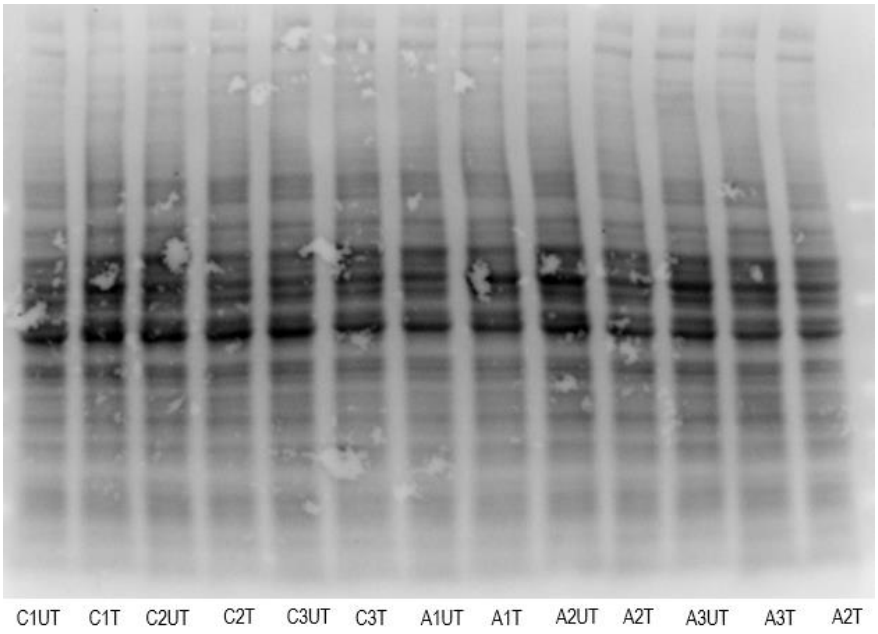
UBQLN2:

MTCO1:



Anexe_WB7

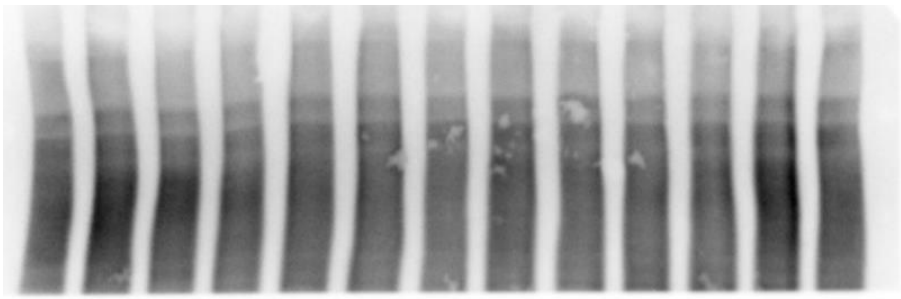
CLQ



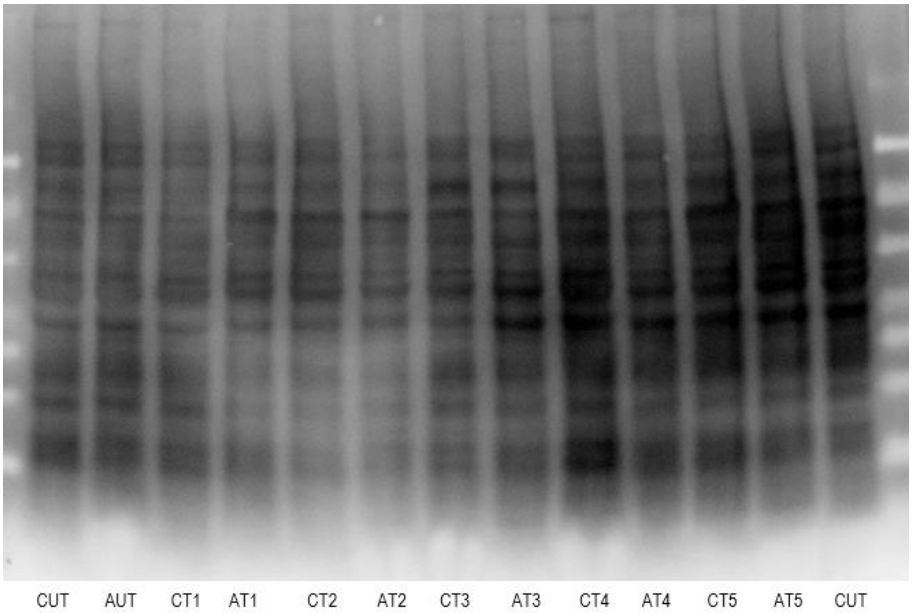
DELE1:

eIF2αP:

Ub:



Anexes_WB8

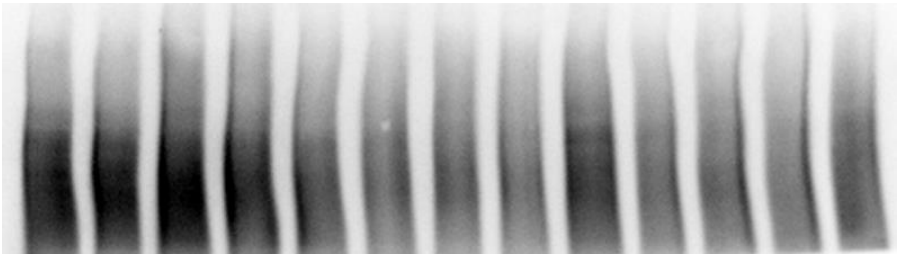


CHOP:

eIF2αP:

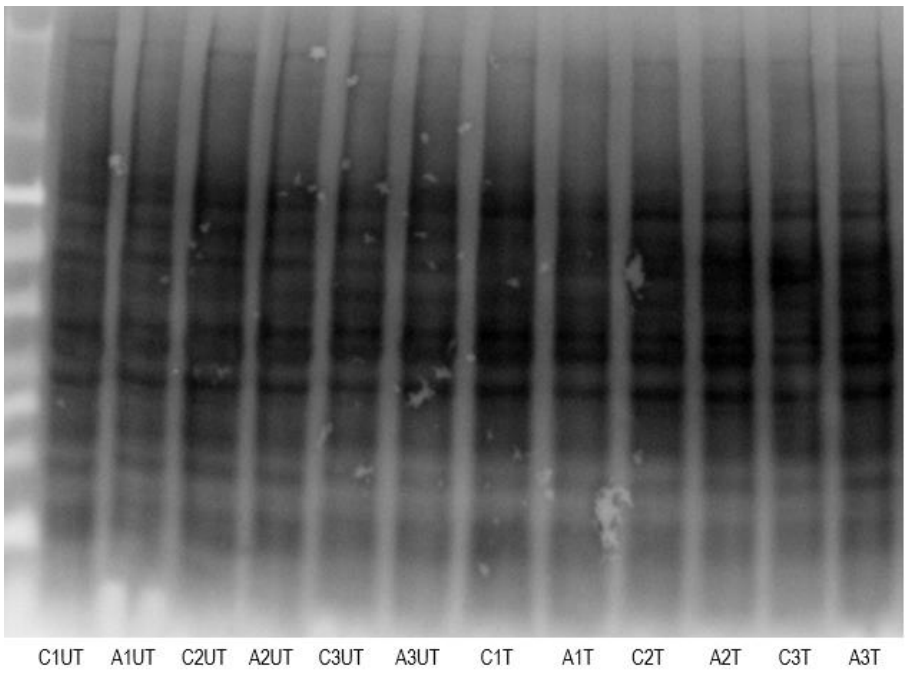
BIP:

Ub:



Anexes_WB9

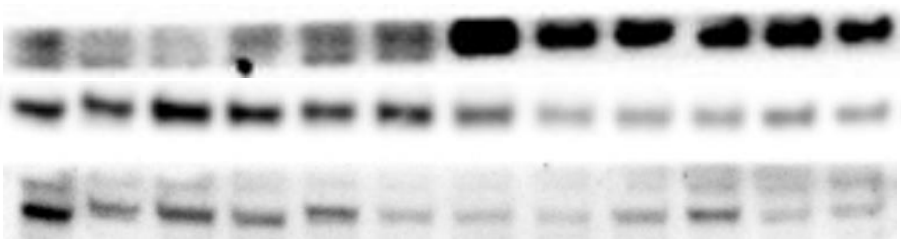
MG132



CHOP:

eIF2αP:

DELE1:



Ub: