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"NEUROPROTECTION BY ESTROGENS IN "IN VITRO" MODEL OF HUNTINGTON'S DISEASE: ROLE OF AUTOPHAGY"

1. Project aim/objectives

Our project aim is to verify whether and how the autophagy is involved in the neuroprotective mechanism of Estrogens, such as 17-β-Estradiol, in Huntington's Disease (HD).

Autophagy is a catabolic process devoted to the lysosomal degradation of self-constituents, that are redundant, aged or have been demaged. Autophagy acts as a survival mechanism under conditions of stress, maintaining cellular integrity by regenerating metabolic precursors and clearing subcellular debris. (Levine and Kroemer 2008; Ravikumar et al. 2010). This process contributes to basal cellular and tissue homeostasis. Moreover, autophagy can provide protection against aging and a number of pathologies such as cancer, neurodegeneration, cardiac disease and infections (Lavallard et al., 2012). Neurodegenerative diseases are age-dependent, hereditary or sporadic disorders that are associated with progressive loss of neuronal function. Common features in their pathogenesis are mitochondrial dysfunction and the accumulation of protein aggregates as a result of mutation and impairment of clearance mechanisms (Jellinger 2010; Goedert et al. 2010). Autophagy is dysregulated in neurodegenerative disorders (Wong and Cuervo 2010). Autophagy starts with the sequestration of target substrates within double-membrane vesiscles named autophagosome, which ultimately fuse with lysosome and endosome to form the autophagolysosome, in which the substrates are degraded by acidic lysosomal cathepsins (Castino et al. 2010). Dysfunctional autophagy compromises the clearance of aggregates-prone mutant proteins that are potentially harmful to neurons, and is therefore considered an important factor in neurodegeneration (Castino et al. 2010). Autophagy exerts beneficial effect to neurons enabling the removal of toxic protein aggregates and oxidized mitochondria, but it may be detrimental if hyper-activated by multiple triggers (Kepp et al. 2011). Thus, while sustained basal autophagy assures cell

homeostasis and it is neuroprotective, either insufficient and frustrated autophagy or sustained up-regulated autophagy may lead to neuronal cell death (Klionsky et al. 2012).
We will test the hypothesis that 17-β-Estradiol exerts a neuroprotective effect against oxidative stress in HD-neurons via estrogens-mediated enhancement of basal autophagy, which promotes the sequestration and lysosomal degradation of toxic huntingtin (Htt) aggregates.

2. Background

Huntington's Disease is a devastating autosomal dominant neurodegenerative condition caused by abnormal expansions of a polyglutamine (polyQ) tract to more than 37 Qs in the huntingtin protein (Rubinzstein 2002). HD affects approximately 5-10 individuals per 100'000. Individuals typically suffer from progressive motor and cognitive impairments, loss of self and spatial awareness, depression, dementia and increased anxiety over the course of 10-20 years before death. Currently, treatment is limited to suppressing chorea: the involuntary and irregular movements of the arms and legs that accompanies HD (Munoz-Sanjuan and Bates 2011). The principal histopathological feature of HD is the loss of medium-sized spiny neurons (MSNs) and, to a lesser degree, neuronal loss in cerebral cortex, thalamus, hippocampus and hypothalamus. Prevalence of HD is equally distributed between both sexes (Sarfarazi et al. 1987; Pearson et al. 1955). Onset is generally in the midlife, but can occur in childhood and old age. At the molecular level, HD is caused by a CAG trinucleotide repeated expansion within exon 1 of the HTT gene. This gives rise to an elongated polyglutamine tract at the amino terminus of the translated huntingtin (Htt) protein that is associated with protein aggregation and gain-of-function toxicity (Davies et al. 1997). In HD, the degeneration preferentially occurs in striatum, deep layers of the cortex and, during the later stages of the disease, extends to a variety of brain regions, including hypothalamus and hippocampus (Vonsattel et al. 1985). Mutant Huntingtin (mHtt) is highly aggregation prone and the formation of cytoplasmatic aggregates and nuclear inclusions throughout the brain is one of the most striking hallmarks of HD (DiFiglia at al. 1997; Soto 2003). The Htt protein is widely expressed within the CNS, more intensely in neurons than in glial cells, and in extraneural tissues. Normal Htt has been shown to be anti-apoptotic (Dragatsis et al. 2000; Ramaswamy et al. 2007; Rigamonti et al. 2000) and essential for normal embryonic development (Duyao et al. 1995; Nasir et al. 1995; Zeitlin et al. 1995). From immunohistochemistry, electron microscopy, and subcellular fractionation studies of the molecule, it has been found that Htt is primarily associated with vesicles and microtubules (Hoffner et al. 2002; DiFiglia 1995). These appear to indicate a functional role in cytoskeletal anchoring or transport of mitochondria. The Htt protein is involved in vesicle trafficking as it interacts with HIP1, a clathrin binding protein, to mediate endocytosis (Velier et al. 1998; Waelter et al. 2001). At

cellular and molecular levels, HD is characterized by the accumulation of polyQ-Htt aggregates, which causes neuronal dysfunction and massive neurodegeneration in the striatum and other brain regions (DiFiglia et al. 1997). Htt protein can be degraded within lysosomes by cathepsin D (Qin et al. 2003).

Mutant Htt toxicity is thought to be exposed after a series of clevange events by proteasomes, calpain and aspartyl proteases, resulting in formation of an N-terminal fragments of around 150 residues containing the polyQ stretch (Sarkar et al. 2008). The length of polyQ influences the toxicity of mHtt. The expanded protein can be cleaved into N-terminal fragments that are more toxic than full-length Htt (DiFiglia et al. 1997; Ratovitski et al. 2007; Ratovitski et al. 2009).

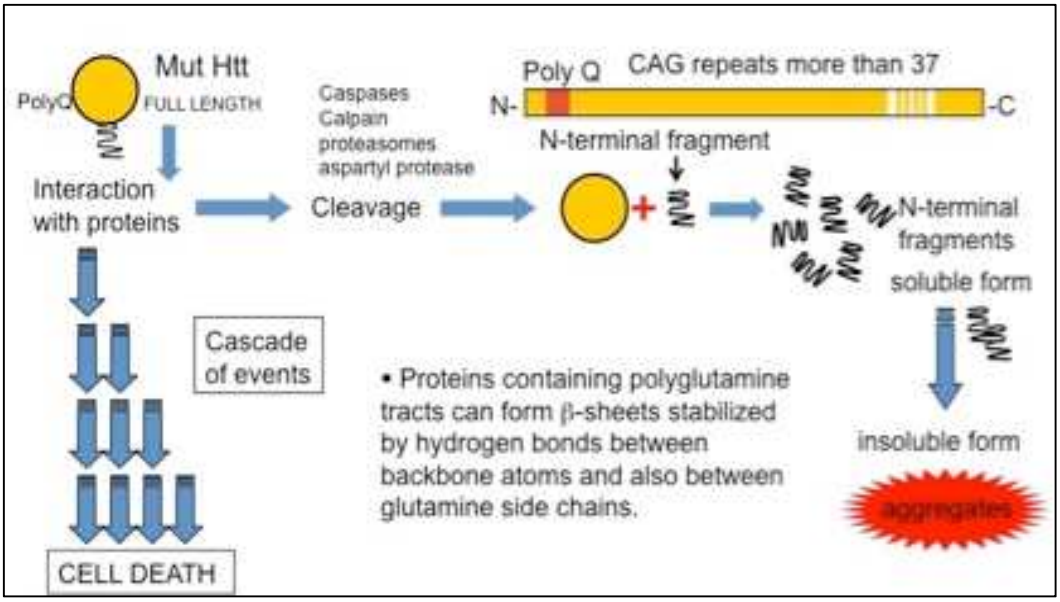


Fig. 1: Scheme of the events leading to cell death in model of Huntington's disease (Rossetti et al. 2008)

The principal HD pathological feature is the loss of striatal and cortical projection neurons. This mechanism remains unclear but may involve excessive glutamate release from cortical and thalamic terminals. Alterations in dopamine (DA) function and neurotransmission have a significant role in motor and cognitive symptoms of HD, as it is well known that glutamate receptor function is modulated by activation of DA receptors. Dopamine plays an essential role in the control of coordinated movements. Modulatory function of DA is disrupted early in disease progression, leading to aberrant glutamate transmission and consequent excitotoxic cascades (Richfield et al. 1991; Van Oostrom et al. 2009). Changes in brain DA content and receptor number contribute to abnormal movements and cognitive deficits in HD. However, later neurochemical studies of HD patients suggested that increased DA occurs in the early stages of the disease (Garrett and Soares da Silva 1992), while post mortem studies of late stage HD patients showed a reduced levels of caudate DA (Kish et al. 1987). Striatal and cortical loss of DA receptor in early stage of HD patients

has been correlated with early cognitive decline, such as attention, executive function, learning and memory (Backman and Farde 2001). Thus, it was thought that DA levels in HD may show biphasic, time-dependent changes, with early increases followed by late decreases associated with biphasic movement symptoms of early HD (chorea) and late HD (akinesia), where in the latter DA levels resemble those of a Parkinsonian-like state (Chen et al. 2012). Although DA exists in high concentration in the striatum, studies also suggest a toxic role for DA in which cell death is accelerated through increases in free radical production (Jakel and Maragos 2000).This has been demonstrated in striatal cultured derived from R6/2 mice, where MSNs undergo DA-mediated oxidative stress and apoptosis (Petersèn et al. 2001 a). DA and glutamate signaling pathways can synergistically enhance MSNs sensitivity to mHtt toxicity (Cepeda et al. 2014).

HD is equally distributed between both sexes (Sarfarazi et al. 1987; Pearson et al. 1955). However, HD is more severe in post-menopausal female as it has been demonstrated that the estrogens and SERMs (Selective Estrogen Receptor Modulators) have protective effects in the brain (Dhandapani and Brann 2002). A few reports focusing on this issue, suggest that the onset age of HD is higher (Roos et al. 1991) and the course of disease is more moderate (Foroud et al. 1999) in women compared with men. Recent clinical studies have highlighted that cellular actions of estrogens in the brain are not restricted to reproductive-related areas, namely hypothalamus, but rather have widespread effects throughout the developing and adult brain (Cyr et al. 2000; McEwen et al. 2002). Indeed, beneficial effects of estrogens are reported in several mental disorders, namely schizophrenia and depression (Cyr et al. 2000; Kulkarn et al. 2002; Osterlund et al. 2005), neurodegenerative diseases such as Alzheimer's and Parkinson's diseases as well as multiple sclerosis and ischemic stroke (Cyr et al. 2000; Shulman et al. 2006; Kompoliti et al. 2003). Although clinical studies provide clear evidence that female sex hormones exert neuroprotective effects in acute and chronic brain diseases, this has not been unequivocally documented in HD (Hoffman et al. 2006; Dhandapani and Brann 2002). Despite of this, it has been demonstrated that transgenic (tg) HD (CAG51) rats, independent of their sex, exhibited increased levels of food intake, elevated home-cage activity scores and anxiolytic-like behavior, whereas only males showed an impairment of motor function. Moreover, this result was associated with a decreased striatal dopamine receptor D1 density and lower plasma levels of 17-β-estradiol; thus, sex hormones exert more powerful neuroprotective effects in female tgHD rats (Bode et al. 2008). Some neurotoxicity protection mediated by 17-β-estradiol has been reported in 3-nitroproprionic acid and quinolinic acid induced models of HD (Tunez et al. 2006; Heron et al. 2000).

Estrogens and estrogen receptors have been implicated in the development or progression of numerous neurodegenerative disorders. Furthermore, the pathogenesis of these diseases has been

associated with disturbances of two key of cellular programs: apoptosis and autophagy. An excess of apoptosis or a defect of autophagy has been implicated in neurodegeneration (Barbati et al. 2012). However, the specific mechanisms of the protective effect induced by 17-β-Estradiol (E2), which is the most efficient estrogens, are not fully understood. E2 is an endogenous gonadal steroid which plays an important role in development, maturation and function of a wide variety of tissue including the nervous tissue. It is known that steroid hormones released from adrenocortex and gonads can pass the blood-brain barrier and bind to intracellular receptors mediating delayed actions with a prolonged duration. It is noteworthy that E2 was recently found to induce autophagy-mediated clearance of ubiquitinated-protein aggregates produced in dopaminergic neuroblastoma cells chronically exposed to a proteasome inhibitor (Isidoro et al. 2009). While high-order aggregated forms of proteins are inefficiently degraded by the proteasome, they can be targeted for degradation by autophagy, a lysosomal degradative pathway (Qin et al. 2003; Rubinsztein et al. 2006).

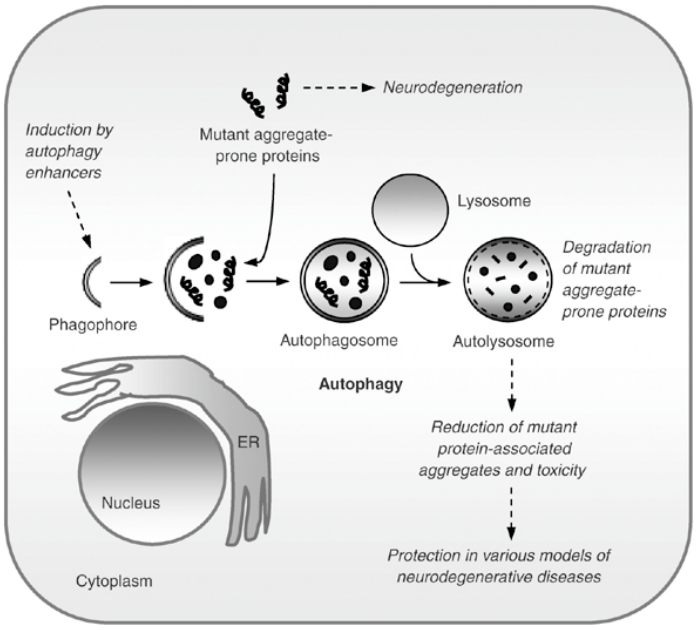


Fig. 2: Autophagy as a protective pathway for the clearance of aggregate-prone proteins. Autophagy is one of the primary degradation pathways for various aggregate-prone proteins associated with neurodegenerative diseases (Sarkar et al. 2009)

The Htt protein itself can act as scaffold to form aggregates that sequester signaling proteins, among which mTOR, a kinase that regulates several pro-survival pathways including autophagy and protein synthesis (Ravikumar et al. 2004). Induction of autophagy reduces both aggregated and soluble Htt species, resulting in a decreased toxicity in HD cell models of fly and mouse (Rubinsztein 2006; Sarkar et al. 2008; Roscic et al. 2011). Of note, enhancing autophagy confers protection in HD animal models (Sarkar et al. 2007). Autophagy has been reported to be up-regulated in post mortem striatum regions of HD patients (Cherra et al. 2010) and autophagosomes

accumulate in primary striatal neurons from HD mice expressing truncated mutant huntingtin following dopamine-stimulated oxidative stress (Petersén et al. 2001 b). More recently, it has been demonstrated that mHtt protein negatively interferes with p62-mediated cargo recognition, thus leading to frustrated autophagy in HD neurons (Martinez-Vicente et al. 2010). Furthermore, early and sustained expression of autophagy-related proteins in a novel knock-in HD mouse model suggests that the autophagic flux alteration is an early and important component of neuronal response to mHtt (Heng et al. 2010).

Based on the premises reported in the background we will test whether estrogens (E2) exert neuroprotective activity toward Huntington's Disease through the enhancement of basal autophagy, which promotes the sequestration and lysosomal degradation of Htt-aggregates.

3. Experimental plan and methods

For our study, the human dopaminergic neuroblastoma SH-SY5Y cell line was employed. For future experiments we will also use human dopaminergic neuroblastoma SH-SY5Y cells engineered to over-express or knocked-down for Cathepsin D. These cells have been produced in our laboratory. SH-SY5Y cells were cultured under standard conditions (37°C; 5% CO2) in 50% Minimum Essential Medium and 50% Ham's Nutrient Mixture F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin-streptomycin solution.

Cells were transfected with these plasmids using LipofectAMINE 3000 reagent according to the manufacturer's protocol (Invitrogen). These constructs were kindly provided by Prof.ssa Francesca Persichetti (DSS).

The ectopic mHtt plasmids expression and toxicity was investigated by Western Blot assay and by Immunofluorescence double-staining of Htt and BAX, respectively.

The autophagy flux was checked through analysis of autophagic markers (LC3 and p62/SQSTM) expression.

Cells have been transfected with an empty plasmid (sham) or with plasmids harbouring either of Htt different forms (subcloned into pcDNAzeo (-)). Constructs N171 Q21 GFP, N171 Q150 GFP, N171 Q21 N/X, N171 Q150 N/X encode the amino-terminal 171 amino acids fragment NcoI/XhoI of human Htt protein, with 21 glutamines, which is the wild-type form, and 150 glutamines, which is the mutant form, respectively. Two of these plasmids generated by inserting a fragment of pGreen Latern, encoding a GFP-tag in frame with the C-terminal of the Htt sequence. Moreover we have other two constructs, Q21 FL and Q113 FL, which encode the full length human Htt protein, with

21 glutamines (wild-type Htt), and 113 glutamines (mHtt), respectively. Q21 FL has FLAG-tag in frame with the N-terminal of Htt.

Effects induced by E2 were monitored to evaluate the increasing of autophagic flux by Western Blot analysis of LC3 and p62/SQSTM.
In the future we will investigate whether this E2 induced autophagy is neuroprotective or not against an oxidative stress.
SH-SY5Y cells were treated with E2 at different concentrations (1nM and 100nM) for different periods, ranging from 24h to 48h. Inhibitors of autophagy, such as 3-methyladenine (3-MA) or Chloroquine (ClQ), were used to understand if the E2 treatment is autophagy-dependent.
Alternatively, autophagy down-regulation will be obtained by genetic silencing of critical autophagy proteins (e.g., Atg7, Beclin-1, Vps34, Akt).
Estradiol activity will be counteracted by fulvestrant (ICI 182,780).

Data and statistical analysis of electrophoresis band densitometry have been performed using VersaDOC Imaging System (Bio-Rad) apparatus, equipped with Quantity One software (Bio-Rad). Microscope observations has been performed with Leica DMI 600 fluorescence microscope (Leica Microsystems AG), equipped with Leica Application Suite software. Imaging processing has been made using ImageJ software.

4. Preliminary data

We have some preliminary data.

1) First, we have set up transfection protocol with the reagent LipofectAMINE 3000, to understand the best working conditions. We have varied the amount of plasmid, reagent and time. The results are shown in the Fig.1. By immunofluorescence analysis, we found that the lower amount of plasmid leads to a low efficiency of trasfection. Immunoblotting of Htt in a time course (36h-48h-60h-72h) experiments shows a decrease of Htt expression in cells transfected with 5 or 7 μg of N171 Q150 DNA. So, the best transfection condition is to use 6 μg of plasmidic DNA and 7,5 μl of LipofectAMINE 3000.

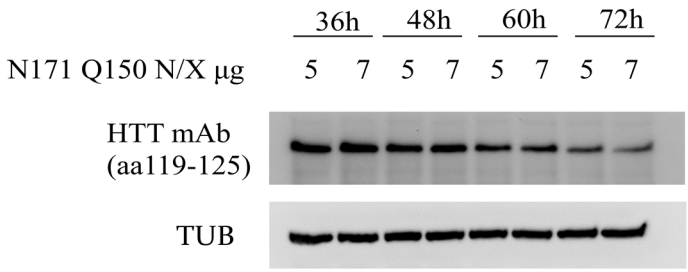


Fig.1: Immunoblotting of HTT mAb (aa 119-125) expression in cells transfected with N171Q150 at different time course

2) SH-SY5Y cells were transfected with the empty plasmid (sham) or the plasmids harboring of mHtt (N171 Q21, N171 Q150, Q21 FL, Q113 FL) and the expression of mHtt protein was assessed by Immunoblotting at 24h, 48h and 72h. Data show that Htt levels increased from 24h to 48h, while decreased at 72h for every plasmid (Fig.2).

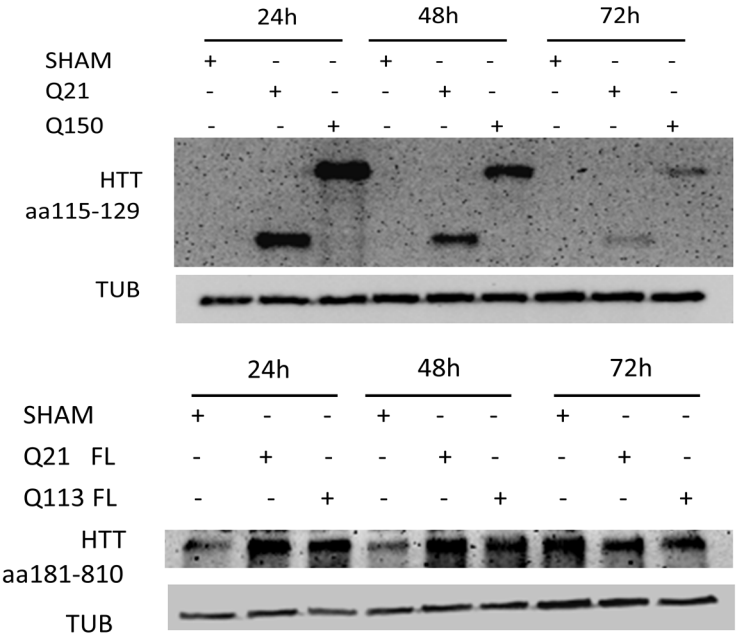


Fig.2: Immunoblotting of ectopic mHtt expression using HTT (aa115-129), which recognizes N-terminal Htt sequence, and HTT (aa181-810), which recognizes full-length protein

3) The toxicity induced by mHtt plasmids transfection was investigated by Immunofluorescence double-staining of Htt in red and BAX in green, respectively, at 48h (Fig.3). This panels show that pro-apoptotic protein BAX expression is evident in cells transfected with N171 Q150 and Q113FL mutants plasmids compared to sham and both wild-type forms, correlating with Htt expression levels. As reported, Htt mAb recognizes both wild-type and mutant forms; for this reason, endogenous Htt protein can be detected in sham condition.

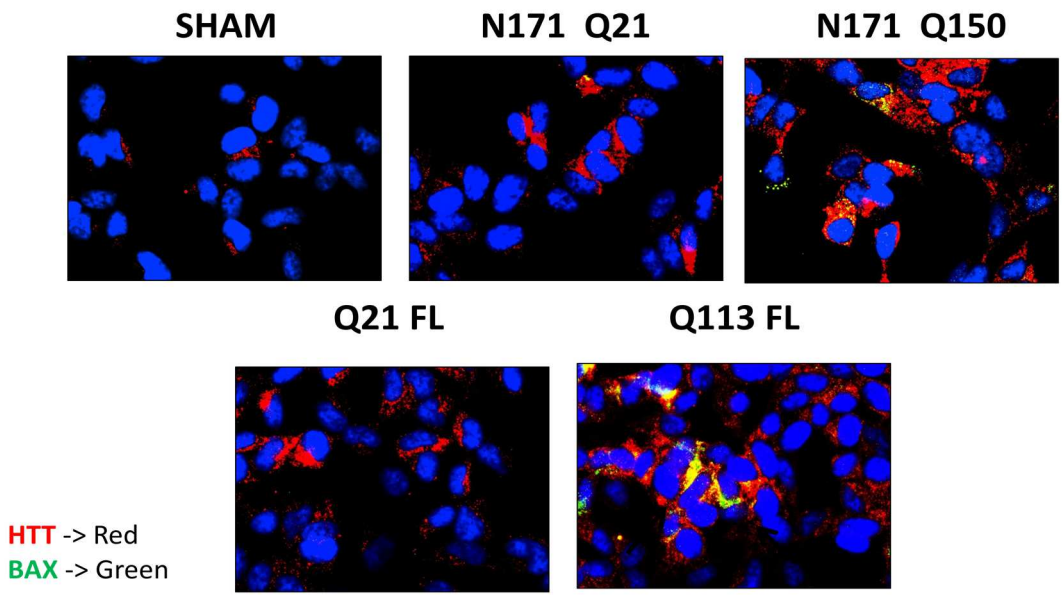
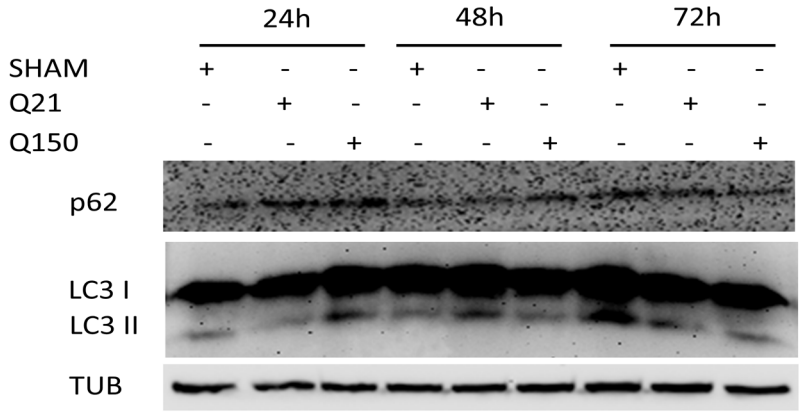


Fig.3:Htt Toxicity by Immunofluorescence double-staining of HTT mAb and BAX

4) We have also analyzed the autophagic flux after transfection with the above plasmids by Western Blot of LC3 and p62/SQSTM (Fig.4). We have observed an accumulation of LC3 and p62 in cells transfected with mHtt plasmid at 24h and 48h; therefore, in particular at 24h, it is evident a block of the autophagic flux; by contrast, at 72h we have observed a reduction of LC3 and p62/SQSTM. These data confirm that autophagy is involved in early transfection time, correlating with Htt expression levels.



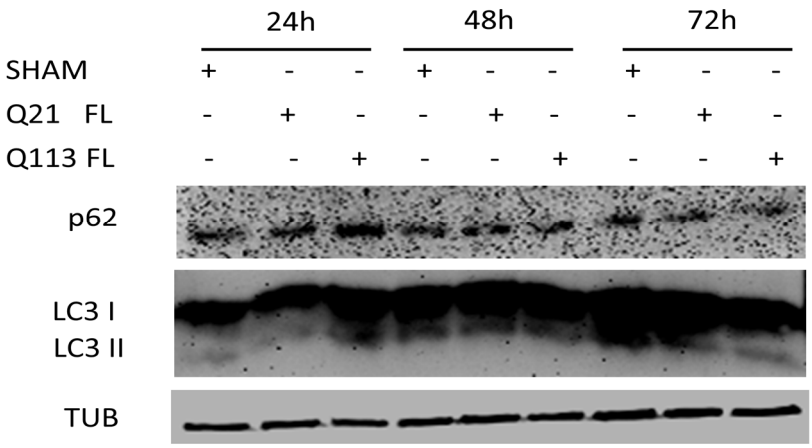


Fig.4: Immunoblotting of LC3 and p62/SQSTM after Htt plasmids transfection

5) We have checked the autophagic flux after E2 treatment using autophagic inhibitors (3-MA and ClQ) by Western Blot analysis. We have treated with two E2 concentrations (1nM and 100nM) at different times (24h-48h). The culture medium was replaced every 16h and 8h, and E2 was re-added. Data demonstrate that LC3 increases after E2 treatment and co-treatment with ClQ, while decreases after co-treatment with E2 and 3-MA (Fig.5). In both co-treatments we have observed an increase of p62/SQSTM. Therefore, E2 induces autophagy.

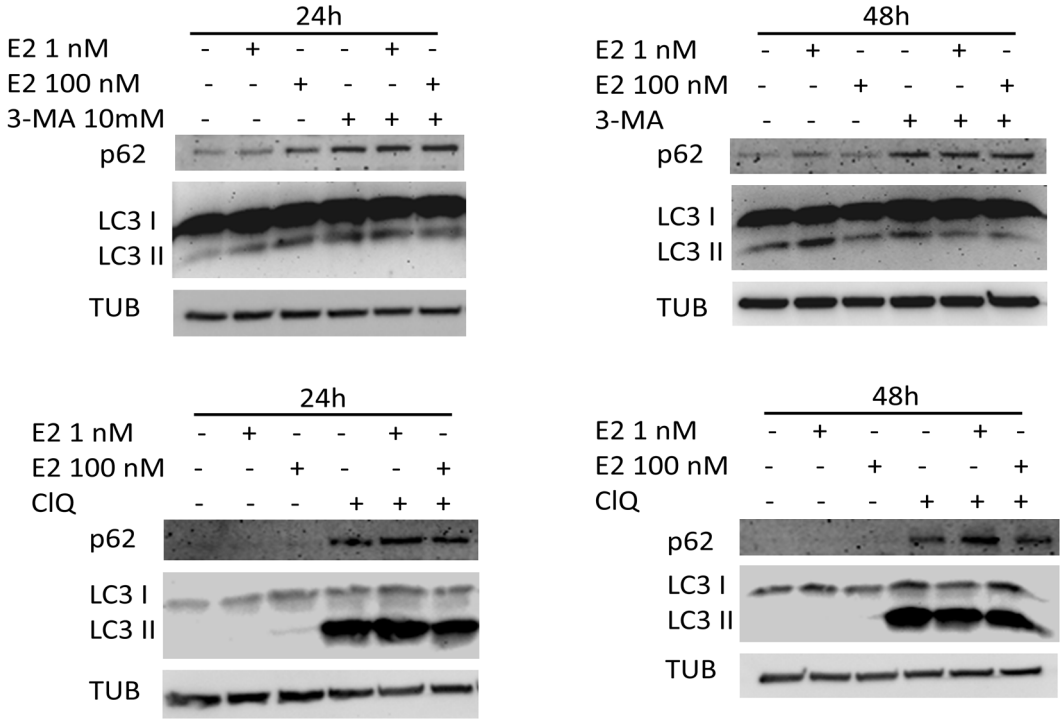


Fig.5: Immunoblotting of LC3 and p62/SQSTM after E2 treatment with autophagic inhibitor (3-MA and ClQ)

In conclusion, our preliminary data provide tools to study neuroprotective activity induced by estrogens in SH-SY5Y cells subjected to oxidative stress after Htt plasmid transfection, through

involvement of autophagy. Understanding if estrogens have neuroprotective activity in HD cell models is fundamental to open new perspectives in HD therapies.

In the future experiments we will evaluate the toxicity induced by ectopic expression of mHtt plasmids under oxidative stress with H_2O_2 or dopamine (DA) by flow cytometric analysis of Annexin V/PI and activation of caspases. Apoptosis will be also morphologically assessed (DAPI staining for chromatin alterations, cell number reduction).
We will use human dopaminergic neuroblastoma SH-SY5Y engineered cells knocked-down for Cathepsin D (CD) to understand if CD is the only lysosomal aspartic protease implicated in degradation of mHtt aggregates.

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B.5b Participation at courses or summer schools organized by others than the PhD program

Course title and location	Organizer(s) and affiliation(s)	Number of hours	
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B.8b

Periods (months) in foreign institutions and laboratories (address)	
Publications (articles, posters, abstracts)	• **Vidoni C**, Titone R, Morani F, Follo C, Isidoro C. Epigenetic control of Autophagy in cancer: the role of microRNAs MINERVA BIOTECNOL • Titone R, Morani F, Follo C, **Vidoni C**, Mezzanzanica D, Isidoro C Epigenetic Control of Autophagy by MicroRNAs in Ovarian Cancer BioMed Research International • Titone R, Morani F, **Vidoni C**, Phadngam S, Valente G, Galetto A, Alabiso O, Isidoro C Autophagy and cancer progression: the role of epigenetics**.** International Symposium on Cell Biology, 4th Ukrainian Congress for Cell Biology (September 17-20, 2014) Uzhgorod, Ukraine

B.XX Attended seminars

Speaker	Title
1. Dr Pietro Lio 5-6 December 2013, 10-12.00 am	Estimating/Understanding Complexity in commodity

17-18 December 2013, 10-12.00 am 8-9 January 2014, 10-12.00 am 20 and 21 January 2014, 10-12.00 am	
2. DR.Dario Colett 10 January 2014 , 14-15.00 p.m	Molecular and Cellular mechanisms f cancer cachexia and treatments
3. Regina Day Professor di farmacologaia alla Medical School della Uniformed ,Universitydi Besthesda 25 November 2013, 14.00 p.m	Isoforms of Hepatocyte Growth Factor : Partial Receptor Activation and differential Expression
4. Chiara Borsotti Culumbia Center for translation Immunology, Columbia University, Nex york 2 October 2013	Humanized mice : A tool for studying in vivo the adaptive immune system development
5. Dr.Ignacio Rubio Friedrich-Schiller-University,Germany 13 January 2014 ,16 .00 pm	Spatial temporal control of RAS activation
6. Dr.Martina Mattii 20 January 2014 ;14.00 pm	Skin as an immune organ responding to external and internal stimuli
7. Prof.Claudio Brancolini University of Udine 30 January 2014	The MEF2-HDACs axis a global regulator of the genetic repromming.
8. Prof.Salvatore Oliviero University of Torino &HuGeF 19 February 2014 , 14.00 pm	Epigenetic modification that control stem cell differentiation
9. Prof. Emilio Hirsch 19 March 2014	Role of Phosphoinositides-3-kinase C2-alpha, a Class II PI 3-kinase, in development and cancer
10. MACS Simposio 12 May 2014	Il ruolo emergente delle vescicole extracellulari in fiopatologia: da mediatori cellular a biomarker
11. Dr. Francesco Barone-Adesi 23 May 2014	Methods for the analysis of the exposure-time-response relationship in epidemiology
12. Dr.Lacopo Baussano Universita del Piemonnte Orientale	Assessment of cervical cancer control in Rwanda and Bhutan

IARC Lione 9 June 2014	
13. Prof.Fabrizio Lorenni 11 June 2014	Ribosome alteration in cancer: effect or cause
14. Prof. Giani Cesareni 12 June 2014	Metformin rewires the signaling netwrk of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli
15. Prof.ssa Antonia Follenzi 19 June 2014	Terapia Genica
16. Prof. Gianni Del Sal 26 June 2014	Disarming mutany p53 in cancer cell
17. Manuela Sironi Scientific Institute e Media Bioinformatics Lab 27 June 2014	Has Nature done the experiment for us? Evolutionary insights into infection disease susceptility and autoimmunity
18. Dr.Diego Cotella 30 June 2014	The C-Value paradox, Junk DNA and Encode
19. Prof.ssa Antonia Follenzi 14 July 2014	Applicazioni Terapia Genica
20. Dr.Maria Giuseppina Milano Istituto do Genotion e Biofisicu,Napoli) 21 July 2014	A function link between ARX and KDMSC GENFS linkd to neuaronal diseases defines a crucial epigenetic disease path
21. Prof. Steven R Ellis 8 September 2014 9 September 2014 10 September 2014 11 September 2014 15 September 2014 16 September 2014 17 September 2014 22 September 2014 10-12.00 am	The Borghese Sessions (SKIN)
17. Dr. Ciardullo Carmela	Clonal evolution and clinical relevance of subclonal

1 October 2014	mutations in chronic lymphocytic leukemia
18. Dr. Famà Rosella 20 October 2014	The Krüppel-like factor 2 transcription factor is a novel tumor suppressor gene recurrently mutated in Splenic Marginal Zone Lymphoma