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Dottorato di Ricerca in Fisiopatologia Neurosensoriale

CHARACTERIZATION OF MOLECULAR ISOFORMS AND ROLE OF THE *SURVIVAL MOTOR NEURON* (*SMN*) IN MOTOR NEURONS DISEASES

BIO/09

TESI DI

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A special thanks goes to my family and Marco.

. . . . . you are weak and will never know love or friendship and

I feel sorry for you. . . .

David Yates, Harry Potter and the order of the phoenix.

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ABSTRACT

The Amyotrophic Lateral Sclerosis (ALS) and the Spinal Muscular Atrophy (SMA) are neurodegenerative disorders characterized by progressive loss of motor neurons.

The SMA is generally caused by homozygous deletion or mutation of the *SMN* gene (1) (2) (3); which encodes for a protein that is ubiquitous and multifunctional and it is highly expressed in the spinal cord (4).

The ALS is a familial or a sporadic disease. The 20% of the cases of the familial ALS is caused by a dominant mutation in the *SOD1* gene (5) (6). In addition *FUS* and *TARDBP* are two other genes involved in this disease (7).

The purpose of my thesis is to study the gene, the isoforms, the subcellular localization and the molecular partners of *SMN* protein.

We studied the *SMN* gene by RFLP-PCR and we discovered that there is not deletion in exon 7 and in exon 8 of this gene.

Therefore, *SMN* is not implicated in the pathogenesis of ALS at genetic level; for this reason we analyzed the *SMN* protein. We chose also two other proteins, *FUS* and *TDP-43* because they have the prerequisites for interacting with *SMN* protein. In fact they have a rich in glycine domain and this is fundamental for the interaction with the *SMN* protein (8). Our studies revealed that the proteins analyzed have different isoforms.

In addition we found that *SMN* and *TDP-43* proteins are both in the nucleus and in the cytoplasm, conversely the *FUS* protein is only in the nucleus.

We subsequently evaluated the interaction of the *SMN* with the *FUS* protein by co-immunoprecipitation. It showed that only a specific isoform of *FUS* interacts with the *SMN* protein and this interaction occurs only in the nucleus.

Then we understood that the localization of the *FUS* and the *SMN* proteins and their interaction does not change during differentiation of neuroblastoma cells (*SH-*

SY5Y) into neuronal-like adult cells by retinoic acid treatment and pretreatment with poly-lysine/poly-ornithine.

Conversely, the localization of the *FUS* protein changes in human fibroblasts, taken from skin biopsy of an asymptomatic subject with *P525L FUS* mutation. In these cells the *FUS* protein is found both in the nucleus and in the cytoplasm.

The translocation of the mutated *FUS* from the nucleus to the cytoplasm has already been discovered by other authors in patients with amyotrophic lateral sclerosis. Here we show, for the first time, that the same phenomenon is present in a subject with *FUS* mutation but asymptomatic.

In conclusion, from all these analyzes, it is possible to deduce that:

- The *SMN* gene deletion is not present in ALS patients.
- *SMN* and *FUS* proteins interact in the nucleus of neuroblastoma cells (*SH-SY5Y*).
- *SMN* and *FUS* proteins maintain the same location in the different cells types analyzed.
- The FUS protein moves itself to the cytoplasm in an asymptomatic subject with *P525L* mutation in the *FUS* gene.

INTRODUCTION

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS).

The amyotrophic lateral sclerosis (ALS) is a multifactorial neurodegenerative disorder characterized by progressive loss of the upper motor neurons and lower motor neurons (figure 1) (1) (2).

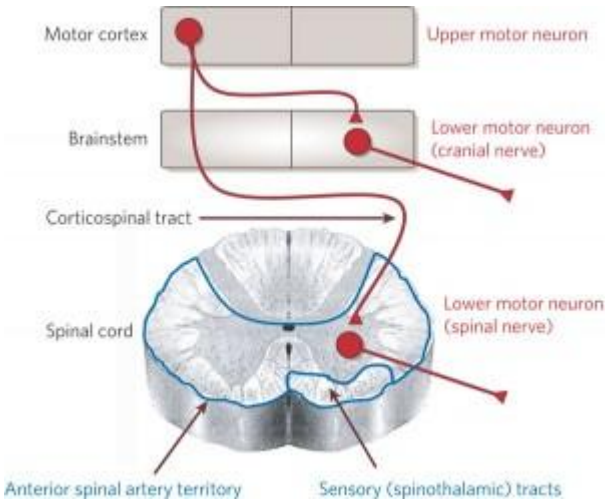

Figure 1: Representation of upper and lower motor neuron.

ALS ‘s pathogenic mechanism is unknown but many factors have been considered, including gene’s mutations, mitochondrial damage, oxidative stress, glutamate excitotoxicity, viral infection, protein aggregation, environmental factors. In addition microglia and astrocytes seem to be involved (9).

The major environmental causes of this disease are: cigarette smoking (10) (11), exposure to heavy metals (12), pesticides (13) and intensive physical activity (14).

In 95% of cases ALS is sporadic (s-ALS) and it is only in 5% of the cases genetically transmitted (f-ALS) (15).

f-ALS can be transmitted in autosomal dominant manner (in which the majority of affected individuals has a sick parent), autosomal recessive manner (rare, the parents of an affected individual are obligate heterozygotes but healthy) or X-linked (rare) (16).

Specifically, the 20% of cases of f-ALS is caused by a dominant mutation in the *SOD1* gene (5) (6), though many genes appear to be involved in the ALS pathogenesis, for example chromosome 9 open reading frame 72 (*C9orf72*), superoxide dismutase-1 (*SOD1*), angiogenin (*ANG*), fused in sarcoma/translated in liposarcoma (*FUS/TLS*), TAR DNAbinding protein 43 (*TARDBP/TDP-43*), vesicle-associated membrane protein B (*VAPB*), optineurin (*OPTN*), valosin-containing protein (*VCP*), ubiquilin-2 (*UBQLN2*), sequestosome-1 (*SQSTM1*), and profilin-1 (*PFN1*) (17).

In addition, some of these genes are involved in the RNA metabolism (18). However, an unsolved enigma is how the disruption of ubiquitously expressed splicing factors can cause selective dysfunction of specific neurons (19).

To solve this question various assumptions have been proposed, including the presence of neuro-specific factors, like *n-PTB* (*polypirimidine binding protein or hnRNP I*) (21) (22) and *NOVA* (*Neuro-Oncological Ventral Antigen*) (22) or the presence of mutations in *splicing sites* of neuro-specific proteins. For example, it was discovered that the amino acid transporter *EAAT2* gene, which protects neurons from glutamate excitotoxicity, is mutated in some cases of ALS, this mutation is in *splicing sites* and it causes loss of the protein and then cell damage (23).

Spinal Muscular Atrophy (SMA) and Survival Motor Neuron (SMN) gene.

The Spinal Muscular Atrophy is a recessive genetic disease with early onset, which affects lower motor neurons and it causes skeletal muscle atrophy. There are different types of SMA based on the onset age and the disease severity (24).

In particular, SMA is caused by homozygous deletion in *SMN1* gene (3) (25).

The *SMN* gene is 27 kb and includes 9 exons (exons 1, 2a, 2b, and 3–8) (27).

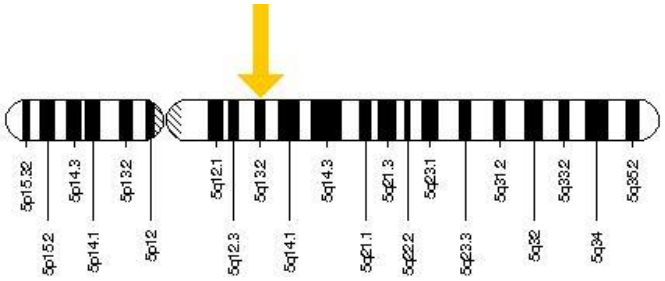

Figure 2: Diagram showing the SMN locus. http://ghr.nlm.nih.gov/gene/SMN1.

In humans, this gene is on chromosome 5q13 (figure 2) and it is present in two copies: *SMN1* (telomeric copy) and *SMN2* (centromeric copy) (3) (26), that have identical sequence, except 5 nucleotides (one in intron 6, one in exon 7, two in intron 7, and one in exon 8) (27) but none of these changes cause modifications in the amino acid of the *SMN* protein (28).

SMN and splicing.

A difference between *SMN1* and *SMN2* genes is the C to T transition in exon 7, that reduces the efficiency of exon 7 inclusion in the *SMN2* transcript, resulting in a truncated protein with altered activity (29) (30).

In fact, in eukaryotes, the immature transcript is produced just after the gene transcription (*pre-mRNA*) and it contains exons and introns. Then the *pre-mRNA* undergo splicing, a process that removes introns and joins exons to produce mature messenger RNA (*mRNA*) (figure 3).

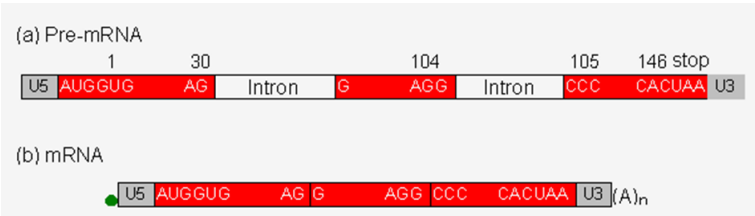

Figure 3: (a) Diagram of a pre-mRNA and (b)a mature mRNA.
www.web books.com/MoBio/Free/Ch5A4.htm.

In *pre-mRNA* the boundary intron-exon is defined by the *splice sites* that are located at the 5 'and 3' of each intron (figure 4) and by an internal signal, *branch site*, characterized by the presence of adenine (figure 4) (31).

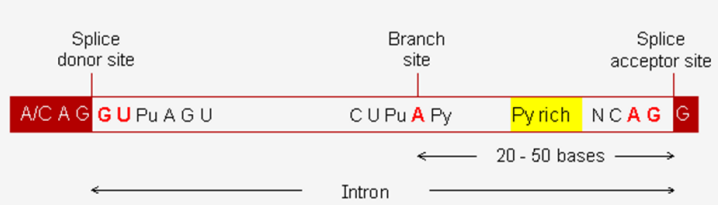

Figure 4: Representation of the splicing sites of a pre-mRNA.
www.web-books.com/MoBio/Free/Ch5A4.htm.

The splicing is catalysed by the *spliceosome*, which contains five small ribonucleoproteins (*U1, U2, U4, U5*, and *U6 snRNPs*) and many other proteins (figure 5) (32).

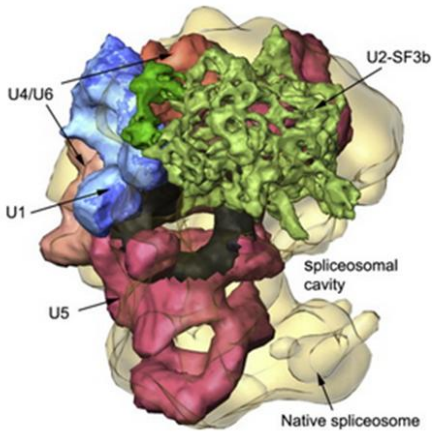

Figure 5: Native Spliceosome model. Ziv Frankenstein et al., 2012.

Except the *ribonucleoprotein U6*, whose biogenesis is thought to be only in the nucleus (33), the other *snRNPs* are transcribed in the nucleus, exported to the cytoplasm and assembled, by the *SMN complex* (34), with the *Sm* proteins (*B/B0, D1, D2, D3, E, F* and *G*) (figure 6) (35).

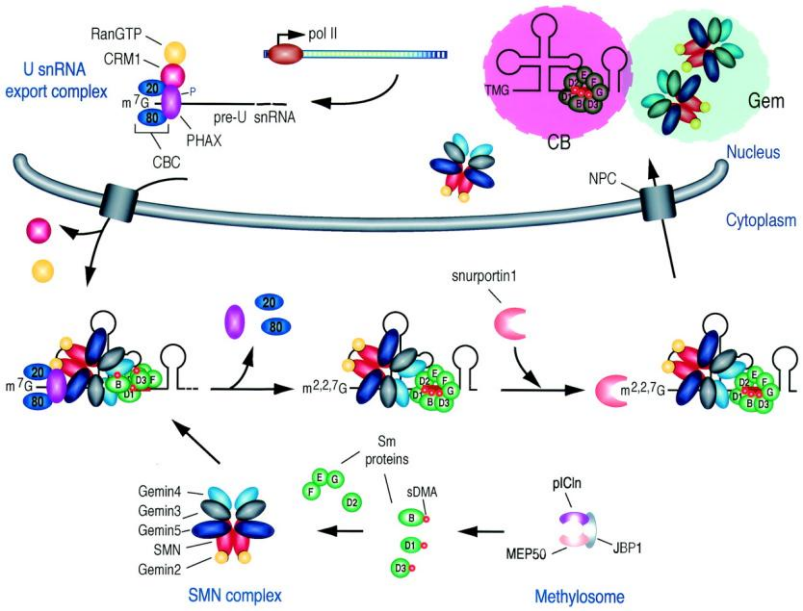

Figure 6: *snRNP* assembly scheme. Séverine Massenet et al., 2002.

Then, the *snRNPs* partially mature (36), return to the nucleus and accumulate themselves in *Cajal Bodies* (*CB*) (figure 6), where they continue their maturation and when this is completed, they move to nuclear *speckles*, in which will remain as a reserve, until the time of splicing (37) (38) (39).

In eukaryotic cells, alternative splicing also occurs in addition to constitutive splicing.

This process is very important in regulating the eukaryotic transcriptome and often, the different *mRNA* isoforms of a same gene in different tissues derived from alternative splicing (40). It is also occurs very frequently in the nervous tissue (41).

Alternative splicing can be done in several ways (figure 7), for example in humans can occur through intron retention, exon skipping (the most common event), 3' alternative splicing, 5' alternative splicing, mutually exclusive exons, alternative first exons, and alternative last exons (42).

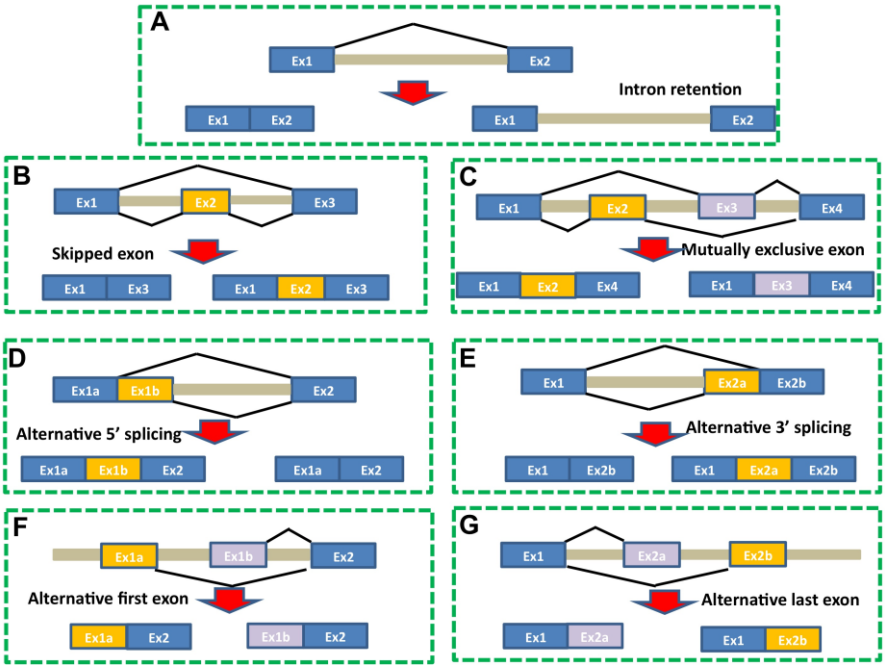

Figure 7: Diagram that representing the different possible events of alternative splicing.
E Z, Wang L, Zhou J., 2013.

Special sequence elements, near the *splice sites*, activate or repress alternative splicing (20) through the recognition of *cis* elements by *trans* factors (figure 8) (40).

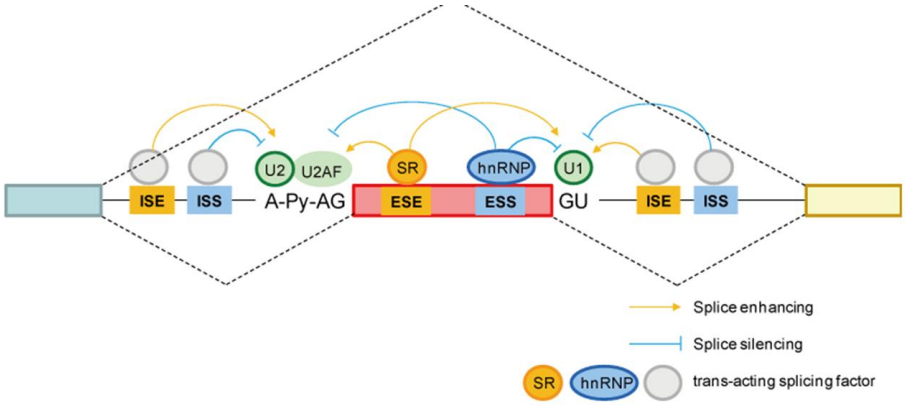

Figure 8: Diagram showing the *cis* regions of transcript and *trans* factors that recognize them.
http://www.h-invitational.jp/h-dbas/as_mechanism.jsp.

In particular, *trans* factors examples are the *SR* proteins, that can cause the non-inclusion of the exons (43) and the *hnRNPs* that often act as repressors (figure 8) (44) (45).

Conversely, the *cis* sequences are (figure 8) Exonic Splicing Enhancers (ESEs), Exonic Splicing Silencers (ESSs), Intronic Splicing Enhancers (ISEs) e Intronic Splicing Silencers (ISSs) (46) (47) (48).

In fact the C/T transition, at position +6 in exon 7 in *SMN2* gene, creates an *exonic splicing silencer* (*ESS*) that is recognized by *hnRNP A1* and this results in the exon 7 non-inclusion (figure 9) (49).

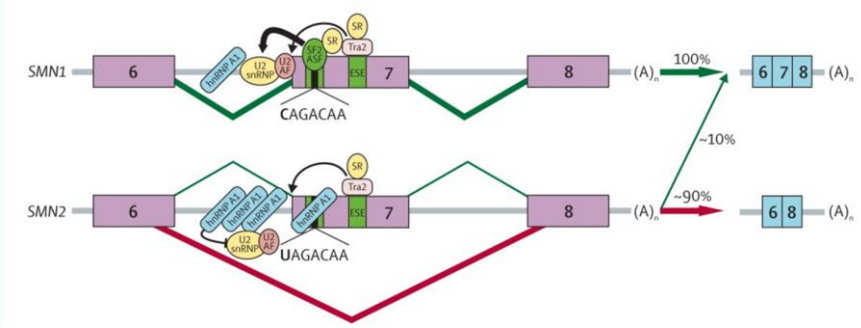

Figure 9: Schematic representation of SMN2 alternative splicing.
www.thelancet.com/journals/lancet/article/PIIS0140673608609216.

This change is the cause of the formation of a shorter protein (*SMNΔ7*) which is less stable (29) (48).

A strong *ESE* is in the central part of exon 7; it is recognized by different splicing factors, that help inclusion of exon 7 (figure 9). This is responsible to *SMN2* full length presence in 10% of cases and this explains why the number of copies of *SMN2* influences the SMA phenotype (50).

Therefore a decrease in *SMN1* protein is present in SMA and specifically in 95% of cases the *SMN1* gene is absent for exon 7 *SMN1* homozygous deletion (51) (27). In 5% of cases other mutations are in the *SMN1* gene, such as small deletions, mutations at sites of splicing and missense mutations (52). These mutations include deletion of 4 or 5 bp in exon 3, an 11 bp duplication in exon 6, five different missense mutations in exons 6 and 7 (53).

The splicing of *SMN2* is also regulated by *hnRNP Q* (figure 10) (54) (56).

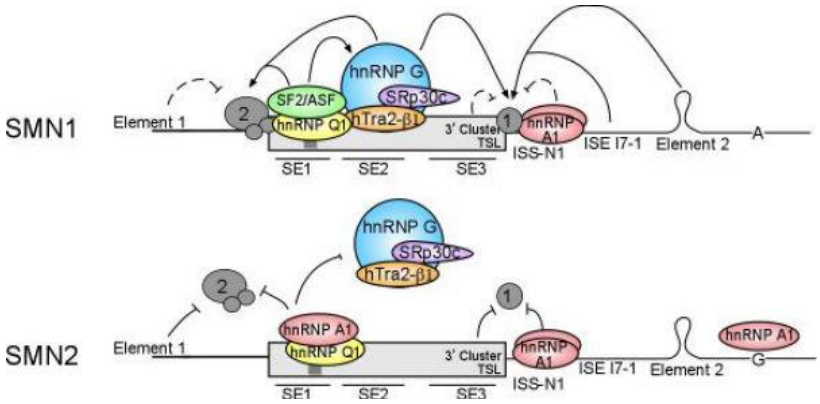

Figure 10: Schematic of some factors that regulate splicing of SMN1 and *SMN2*.
Thomas W. Bebee, Jordan T. Gladman, and Dawn S. Ch, 2011.

The *SMN* protein links itself and it maintains this ability in case of mild mutations. Conversely, in case of exon 7 loss and missense mutations in exon 6, it loses this function, causing severe SMA phenotype (56). This happens because *SMN* is not able to oligomerize and it is immediately degraded and this causes low levels of the protein (57).

The correction of *SMN2* splicing to increase the levels of *SMN2* exon 7 inclusion can be achieved by several strategies, for exsample antisense oligonucleotides and trans-splicing.

Splicing of *SMN* is also regulated by *TDP-43* protein, in fact, it has been found that if *TDP-43* decreases the *SMN* lacking exon 7 increases (58).

Finally, in a recent study, it was shown that in human neuronal and non-neuronal cells, both the *SMN2* and the *SMN1* gene, have different isoforms of transcripts, that are formed by different alternative splicing events that cause, for example, the 3 or 5 exon non-inclusion, thus these transcripts are smaller than the full *SMN* transcript (30).

SMN protein.

SMN is a protein of 294 amino acids. It contains distinct functional domains that mediate various interactions, including interactions with itself, with the *Sm* proteins, with other proteins and with nucleic acids (figure 11) (59).

Figure 11: Representation of the SMN exons and S*MN* domains.
A H M Burghes and C E Beattie, 2009.

As it is possible see in the figure 11, the Tudor domain of *SMN* protein is encoded by exon 3. This domain has been found in several proteins and in *SMN* protein it is required to interact with the *Sm* proteins (60).

The region of *SMN* encoded by exon 2 (figure 11) is necessary and sufficient to mediate its nucleic acid-binding activities, to interact with *Gemin2* and it participates to self-oligomerization (56) (61) (62).

Conversely, the 6 and 7 *SMN* exons contain the information for the formation of the portion of the protein required for self-oligomerization (57) (figure 11) .

The *SMN* protein is part of *SMN-COMPLEX* (figure 12). This complex is formed by *SMN* protein and other 7 proteins (*Gemin2-8*) and it is very important for the biogenesis of specific *snRNP* (63).

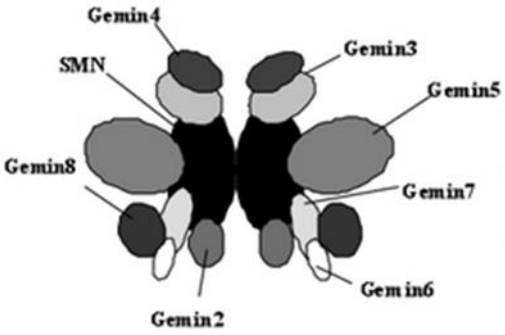

Figure 12: Representation of SMN COMPLEX. Stephanne Levin et al., 2010.

SMN protein interacts with *Sm* proteins through its Tudor domain (64) and it binds specifically them to *snRNP* (figure 13) (65).

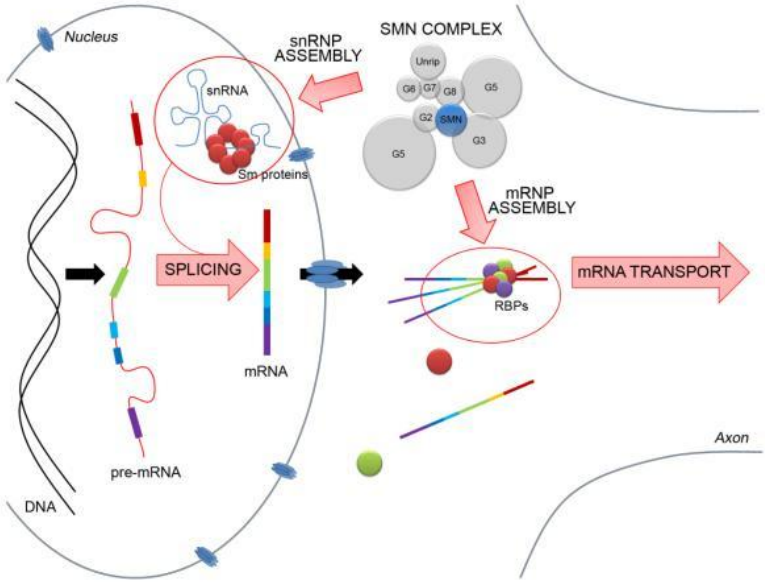

Figure 13: Image that explains the different functions of the SMN protein in neurons.
C Fallini, G J Bassell and W Rossol, 2012.

A recent study has suggested that *FUS* mutations have a significant effect on *snRNP* localization (66).

Furthermore, the assembly of *snRNP* depends on the *SMN* amount present and it is the reason that SMA individuals have a decreased ability to assemble these ribonucleoprotein (67).

In addition *Zhang Z. et al*. have shown that a loss of *SMN* level greater than 80% is necessary to cause a significant change in the levels of s*nRNAs* or cause the death of cells in culture (68).

It has also been shown that *siRNA* silencing of *SMN* has an effect on the *snRNAs U2*, *U12* and *U4atac* and the same happens in SMA patients (69), however it is not clear what *SMN*-mediated mechanism causes localized damage in motor neurons.

To understand this localized effect, *Lotti F*. analyzed the role of splicing U12-*SMN* dependent, in the regulation of motor circuits. This study was done in Drosophila, which has a gene, *stasimon*, that has U12 introns. This gene is a *SMN* target and it

encodes to a protein necessary to a normal synapse in cholinergic neurons. The reduction of *SMN* protein has an effect on *stasimon* splicing and then on its expression in motor circuits. This could explain the localized effect in motor neurons. *Stasimon*'s homologous genes have been found in human, mouse, zebrafish and other species (19).

Moreover, each cell has specific splicing factors and therefore the decrease of *SMN* may have a unique effect on *snRNP*s in each case (68).

Janina Rafatoska et al. analyzed the same problem in amyotrophic lateral sclerosis and they gave an explanation for the lack of effect of protection by the *SMN,* assuming that other components of the *SMN complex* may be involved, such as *gemin* proteins (70).

Wan L. et al. discovered that *ROS* (reactive oxygen species) inhibit the *SMN complex* activity in a dose dependent manner (71) and oxidative stress is one of the possible causes of amyotrophic lateral sclerosis (ALS).

The *SMN* protein was also found in the axon (figure 13) (72) and it is not associated with the *Sm* proteins here then it probably has a different function (73).

TAR DNA-binding protein of 43 kDa (TDP 43) and RNA binding protein fused in sarcoma/translated in liposarcoma (FUS/TLS).

The *TARDBP* and the *FUS/TLS* genes, are mutated in some cases of ALS (74) (75) and they form cytoplasmic aggregates (granules) through independent mechanisms (76).

Missense mutations in *TARDBP*, the gene encoding *TDP-43*, are a known cause of FTLD and of familial and sporadic ALS, ~5% of ALS (77) (78) (79).

The human *TDP-43* gene, which is located on chromosome 1 and contains 6 exons, is alternatively spliced to generate at least four isoforms.

The *TDP-43* protein has 414 amino acid. It contains two highly conserved RNA recognition motifs (*RRM1* and *RRM2*) and a glycine-rich C-terminal sequence (figure

14). This last region mediates the TDP-43 protein interactions (80) whereas the *RRMs* domains mediate RNA recognition (81).

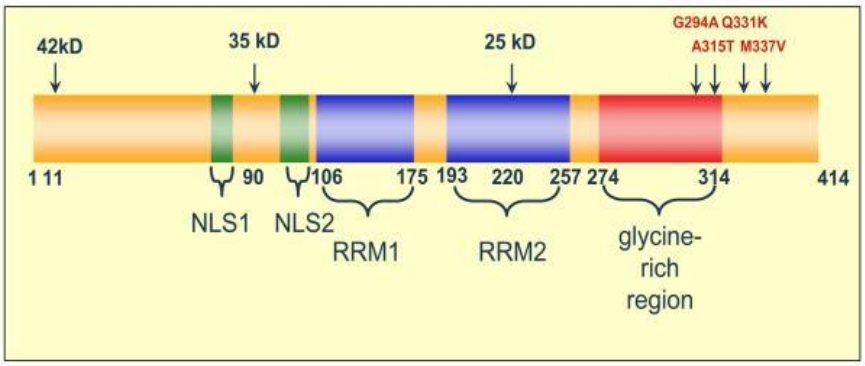

Figure 14: Diagram of the domains of the TDP 43 protein.
Casey Cook et al., 2008.

The *TDP-43* protein interacts with many proteins and this suggests that it is a multifunctional protein, mostly associated with transcription, splicing and translation (82) (80).

For example, it acts as a transcriptional repressor and inhibits exon skipping through the specific recruitment to 3' *splice sites* (83). It was also discovered that regulates alternative splicing of its own transcript (81).

The *FUS* gene is mutated in ~4% of familial and rare sporadic ALS cases (79). Most mutations are missense mutations except for example, a *de novo* splice-site mutation that leading to the skipping of exon 14 and this products a truncated *FUS/TLS* protein (*p.G466VfsX14*) (84).

FUS is a protein formed by 526 amino acid and encoded by 15 exons. It is composed of an N-terminal domain enriched in glutamine, glycine, serine and tyrosine residues (*QGSY region*), a glycine-rich region, an *RRM* domain, multiple arginine/glycine/glycine (*RGG*) repeats in an arginine and glycine-rich region and a C-terminal zinc finger motif (figure 15) (85).

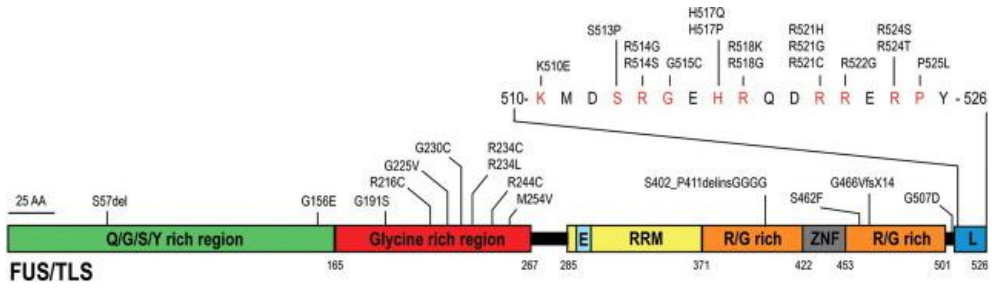

Figure 15: Diagram of the domains of the *FUS* protein.
Clotilde Lagier-Tourenne et al., 2010.

The *FUS* protein interacts with *SMN* protein in mouse neurons and in *HeLa* cells. Mutations in *FUS* would have affect on this interaction causing axon defects (85) (86).

Furthermore it was shown that in case of *FUS* mutation (*R521C FUS*), the *FUS* protein undergoes a relocalization, in primary cultures of human fibroblasts of ALS patients, accumulating itself in the cytoplasm (87).

Neuronal cytoplasmic protein aggregation and defective *RNA* metabolism thus appear to be common pathogenic mechanisms involved in ALS.

RESEARCH OBJECTIVES.

As it was mentioned in the introduction, the amyotrophic lateral sclerosis (ALS) pathogenesis is not yet completely clear.

So the PhD research aim was to provide a further contribution to the knowledge on the ALS molecular basis, with particular attention to the role of *SMN*.

All studies were done at the Neurochemistry Laboratory, Department of Experimental Biomedicine and Clinical Neurosciences, University Hospital "Paolo Giaccone" in Palermo.

The research program was:

- Study of the *SMN* gene through PCR-RFLP in patients with ALS and in control subjects.
- Molecular characterization of the *SMN* protein isoforms to understand whether specific isoforms are involved in the ALS pathogenesis.
- Characterization of *FUS* and *TDP-43* proteins, involved in ALS and that they are *SMN* potential interactors.
- Analysis of the *SMN*, *FUS* and *TDP-43* proteins localization to study if any changes occur in case of particular conditions such as differentiation or mutations.
- Study of the *SMN* interactions with wild type and mutant *FUS* protein to understand if a change of this condition can help trigger the ALS.

CHAPTER-1

CHAPTER 1- *SMN* GENE ANALYSIS.

Introduction and objectives.

We decided to analyze *SMN* gene because in literature there are many studies on this topic. These antecedent studies were done on patients with spinal muscular atrophy (SMA).

As shown in figure 16, the two copies of *SMN* gene are identical except 5 nucleotides (88) and these nucleotide changes are exploited by the PCR-RFLP technique.

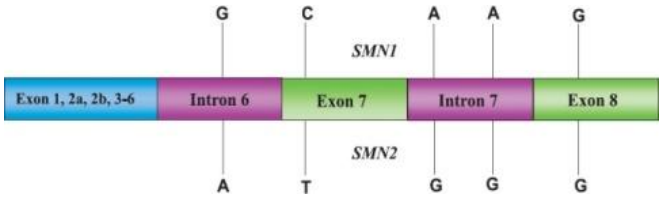

Figure 16: Representation of the nucleotide differences between the *SMN1* and *SMN 2* genes. Balraj Mittal, 2012.

Specifically, PCR products of *exon 8* from *SMN1* and *SMN2* genes were distinguishable by the presence of the recognition site for the restriction enzyme *DdeI* (figure 17).In fact this site is absent in *SMN1* gene but is present in *SMN2* gene.

Conversely, for the recognition of *exon 7* of *SMN1* and *SMN2* genes, a mismatched downstream oligonucleotide primer is used. It recognizes the region adjacent to the *SMN2* exon 7 variant site (figure 17). This creates a *restriction site*, that is recognized by the enzyme *DraI*. In particular, how is shown in figure 17, the change creates a *Dra I* site (*TTTAAA*) because the third T nucleotide of the sequence is present only in the *SMN2* gene, thus the enzyme *Dra I* cuts only the *SMN2* amplified PCR product (89) (90).

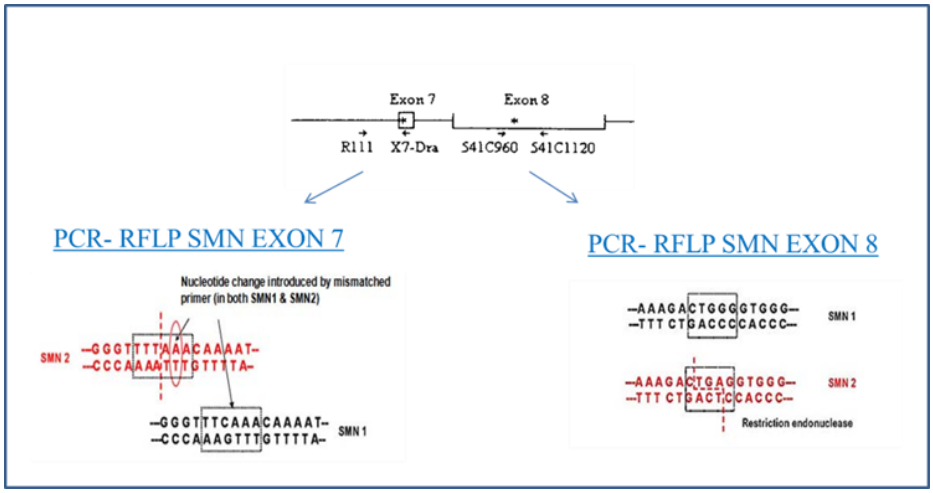

Figure 17: Representation *SMN* 7 and 8 exons RFLP-PCR specific method.
Guey-Jen Lee-Chen et al., 1996.

Numerous studies have been done to understand the role of *SMN* in amyotrophic lateral sclerosis.

For example, *Veldink JH et. al*., in 2001, analyzed deletions of 7 and 8 exons of *SMN1* and *SMN2* genes in sporadic amyotrophic lateral sclerosis (ALS). They showed the presence of a homozygous *SMN2* deletion. Then they suggested that the *SMN2* gene can act as a prognostic factor because patients with a homozygous *SMN2* deletion had a shorter median time of survival (91).

Corcia P. et al. in 2002, studied the same topic and they discovered that 16% of amyotrophic lateral sclerosis patients had an abnormal copy number of the *SMN1* gene (1 or 3 copies) (92). Then in 2006, they explored the frequency of abnormal *SMN1* gene copy numbers, advancing the conclusion that abnormal *SMN1* gene copy numbers is a genetic risk factor in ALS (93).

After a couple of years, we studied the *SMN1* and the *SMN2* genes, by PCR-RFLP method, considering the *Lefebvre* and *Van Der Steege* protocols.

Materials and Methods.

SAMPLES COLLECTION: In our laboratory 46 whole blood samples in tubes with EDTA have been collected. Specifically, we collected 31 ALS samples, 2 SMA samples and 13 neurological controls samples.

PCR-RFLP METHOD: Genomic DNA was extracted from peripheral blood using *Puregene* kit .

Polymerase chain reaction (PCR) amplification of 7 and 8 *SMN* exons was done using 250 ng DNA, preparing from time to time an appropriate mix and executing a different amplification program, based on the type of exon that we wanted to amplify.

In particular, the mix was prepared using 0.5 U Taq polymerase (Euroclone), 200 µM dNTPs (Euroclone), 50 mM $MgCl_2$ (Euroclone), 1X reaction buffer magnesium free (Euroclone), 100 pmol primers specific (Amersham pharmacia biotech), all diluted with water to obtain a final volume of 20 µL.

To amplify the 7 *SMN* exon we used the specific primers (figure 17):

- forward intron 6 primer *R111*:*5'-AGACTATCAACTTAATTTCTGATCA-3'*(94);
- reverse mismatch primer *X7-Dra*: *5'-CCTTCCTTCTTTTTGATTTTGTTT-3'* (95).

The PCR performed by 35 cycles , using a specific amplification program:

initial denaturation: temperature was set to 94 °C for 4 minutes;

denaturation: temperature was set to 94 °C for 1 minute;

annealing: temperature was set to 55 °C for 1 minute;

elongation: temperature was set to 72 °C for 1 minute;

final elongation: temperature was set to 72 °C for 7 minutes.

To amplify the 8 *SMN* exon we used the specific primers (figure 17):

- forward primer *541C960: 5'- GTA ATA ACC AAA TGC AAT GTG AA - 3';*
- reverse primer *541C1120: 5'- CTACAACACCCTTCTCACAG - 3'*.

The PCR performed by 35 cycles, using a specific amplification program:

initial denaturation: temperature was set to 94 °C for 4 minutes;

denaturation: temperature was set to 94 °C for 1 minute;

annealing: temperature was set to 59 °C for 1 minute;

elongation: temperature was set to 72 °C for 1 minute;

final elongation: temperature was set to 72 °C for 7 minutes.

The amplified products were digested, overnight at 37 °C, with appropriate restriction enzymes and buffer, specifically we used :

- *Dra I* and NE Buffer 4 (BioLabs) for 7 *SMN* exon;
- *Dde I* and NE Buffer 3 (BioLabs) for 8 *SMN* exon.

The after day , the digested products were electrophoresed on 2% agarose gel for further analysis.

Results and discussion.

All experiments were performed using ALS samples, positive control (SMA subject), neurological control (patient with neurologic disease but it does not involving motor neurons) and negative control (there was not DNA but water).

The results of the study on 7 *SMN* exon reflect the below image (figure 18). Many experiments have been and in each the positive control, always appeared a single band (176 bp), corresponding to *SMN2* gene. However two bands always appeared that correspond to *SMN1* (188 bp) and *SMN2* (176 bp) in each neurological controls and in each ALS patients.

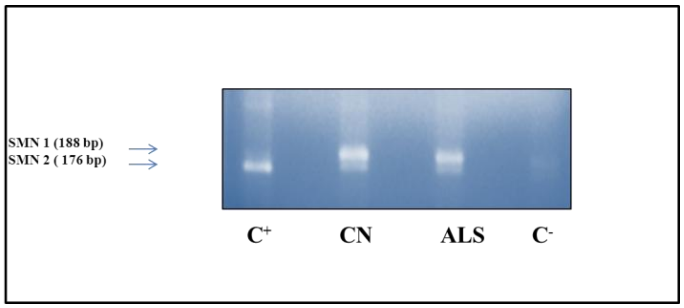

Figure 18: Result experiment of 7 *SMN* exon RFLP - PCR.
C^+ = positive control; CN = neurological control; ALS = patient with ALS; C^- = negative control.

The figure 19 shows the result obtained from the study of 8 *SMN* exon.

In the positive control always appeared two bands (122 bp and 78 bp), which correspond to the *SMN2* gene, conversely always three bands (200 bp; 122 bp and 78 bp) are in neurological controls and in ALS patients, that correspond to the *SMN1* and *SMN 2* genes (figure 19).

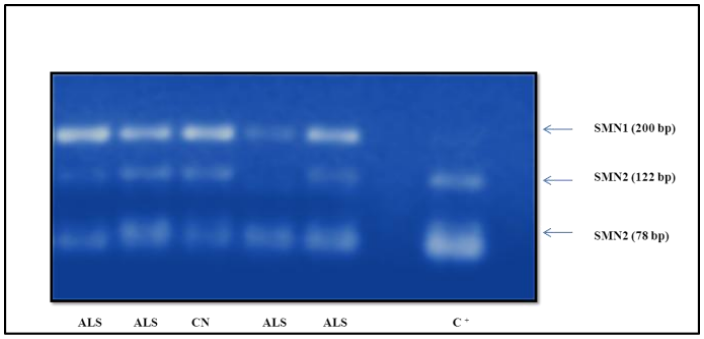

Figure 19: Result experiment of 8 SMN exon RFLP - PCR.
C+ = positive control; CN = neurological control; ALS = patient with ALS; C - = negative control.

All results are summarized in the following table (tab 1).

PATIENTS ANALYZED		EXON 7 SMN	EXON 8 SMN
ALS	31	NO DELETION SMN1	NO DELETION SMN1
C +	2	DELETION SMN1	DELETION SMN1
CN	13	NO DELETION SMN1	NO DELETION SMN1

Tab. 1: Summary of the results obtained from the study of 7 and 8 *SMN* exons with RFLP-PCR.
C+ = positive control; CN = neurological control; ALS = patient with ALS.

Conclusions.

The results obtained from the analysis of SMA patients, that we used as positive control, correspond to the data available in the literature (94). In fact, all SMA patients analyzed show deletion of the *SMN1* gene but this deletion is never present in neurological control and in ALS samples. Conversely the *SMN2* gene is present in all samples.

Therefore, these data indicate that the *SMN1* gene though involved in the SMA pathogenesis, it is not involved in ALS. Then, we done a further contribution to understand the molecular basis of this disease. In fact, there are still conflicting ideas about the role of the SMN gene.

In the future, it would interesting to see if the number of copies of *SMN* genes varies or if there are variations in their promoter that could alter the transcription and then cause disease.

CHAPTER-2

CHAPTER 2- RESEARCH OF ISOFORMS OF *SMN*, *FUS* AND *TDP43* PROTEINS IN HUMAN LEUKOCYTES, *HeLa* CELLS AND *SH-SY5Y* CELLS.

Introduction and objectives.

We studied the different isoforms of *SMN*, *FUS*, *TDP-43* proteins to have a starting point to carry out more specific surveys for each isoform.

The *SMN* is a ubiquitous protein that is found in both the cytoplasm and the nucleus of the cells and which has different isoforms in different cell types (96) (97).

La Bella V. et al. in 2000, showed that two isoforms of *SMN* arise in primary brain neurons of rat and in motor neurons. These have molecular mass of 32 kDa and of 35 kDa. Conversely, in mouse *3T3* fibroblast there is only a 42 kDa *SMN* isoform. Then, they affirmed that the different isoforms have a different subcellular localization and specifically the 32 kDa isoform is in citoplasm (97). The same group in 2006 explained that the smaller isoform is derived by proteolytic cleavage (98).

Many studies were done on the *SMN* isoforms and a lot of them have been identified isoforms with molecular mass between 45 kDa and 24 kDa. In addition the expression of these isoforms changes during the development in various tissues in rat (99).

Sossi V. et al., conducted studies on the same issue, suggesting that the *SMN* RNA isoform that lost the exon 3 produces a protein lacking 67 amino acids, which has a molecular mass about 31 kDa.

An axonal *SMN* isoform (a-SMN) was also discovered. It is an alternatively spliced *SMN* form that is preferentially encoded, in humans, by the *SMN1* gene. In addition 2b and 3 exons are important for its formation. Its molecular mass is less than that of the canonical *SMN* protein (100).

As it has been already described in the introduction, *TDP- 43* protein found mutated in sporadic and familial ALS.

It was shown that, a *TDP-43* isoform with a slightly smaller a *TDP-43* isoform with a slightly smaller molecular mass is in the cytoplasm of leukocytes of ALS patients. However the same levels were found both in the whole cell lysates of the ALS patients and of the controls (101).

Guo W et al, conversely found an greater molecular mass isoform (about 74 kDa) compared to that expected in brain of subjects with frontotemporal lobar degeneration (*FTLD*), other condition in which *TDP-43* has mutated (102).

Finally, a very small isoform of *TDP43* protein, about 25 KDa was found. It thought to be toxic (103) (104). In addition the 35-kDa *TDP-43* isoforms is assembled in stress granules, cellular structures that package mRNA and RNA-binding proteins during cell stress (105).

Mutations in the *FUS* gene are also associated with ALS. The *FUS* protein was detected by western blot as an approximately 72 kDa protein in both human and mouse brains (106).

Materials and methods.

LEUKOCYTE COLLECTION: In our laboratory whole blood samples in EDTA tubes has been collected, the leukocytes were extracted from each fresh sample immediately.

The leukocytes extraction was done by a precipitation with 3% dextran for one hour at room temperature and by centrifuging for 10 minutes at 3500 RPM. Then the pellet was resuspended in cold 0.9% NaCl and it centrifuged for 8 minutes at 3500 RPM. Later the pellet was resuspended with a cold solution of 0,05% KCl and it centrifuged for 8 minutes at 3500 RPM, so many times up to have a clean pellet, once this accomplished, it was resuspended in cold 0,05% KCl and centrifuged for 5 minutes to 5000 RPM. The pellet was used immediately or was stored at - 80 °C until use.

CELL CULTURE: *HeLa* (Human epithelial carcinoma cell line) cells were grown in 75 cm^2 Falcon flask filled in Dulbecco's modified Eagle's medium (DMEM, Euroclone) with 10% fetal calf serum (Euroclone), 2 mM L-glutamine (Euroclone) , 100 U/ penicillin and 100 µg streptomycin (Euroclone).

SH-SY5Y (human neuroblastoma) cells were grown in a 75 cm^2 Falcon flask filled in Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with Nutrient Mixture F-12 (1:1, GIBCO) and with 10% fetal calf serum (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ penicillin and 100 µg streptomycin (Euroclone).

PROTEIN EXTRACTION : The lysis of leukocytes and the lysis of the cells in culture was done on ice, using a specific buffer (10 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.2% SDS) with protease inhibitors (pepstatin, antipaina, leupeptin and PMSF). Then the cell suspension has undergone a brief sonication and it centrifuged at 5000 RPM for 5 minutes at 4 °C. Then the supernatant was recovered.

SUBCELLULAR FRACTIONATION OF CELLS: To make this process we used the digitonin 0.04%, it was resuspended in RSB 100 buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$).

The digitonin stock solution was dissolved in ethanol because the alcoholic solution of digitonin forms insoluble complexes with cholesterol (plasma membrane) but it does not form these with cholesterol esters and then it preserves the nuclei.

The samples were kept on ice in all stages of this process. In particular, the culture medium was aspirated from the cells and they were washed with D-PBS Dulbecco's Phosphate Buffered Saline (Euroclone). Then these cells or leukocites are resuspended in digitonin 0.04%, supplemented with protease inhibitors (pepstatin, antipaina, leupeptin and PMSF) and they were incubated for 10 minutes at 4 °C in constant shaking. Afterward the cells were lysed by repeated passages in 25 G needle and then they were centrifuged at 900 g for 10 minutes at 4 C. The pellet obtained corresponded to the nuclei and the supernatant corresponded to the cytoplasm. Later the nuclei were observed with optical microscope using a 40 X objective (figure 20) and then they were resuspended in RSB 100 buffer with inhibitors of protease. Protease inhibitors were only added in the cytoplasmic fraction.

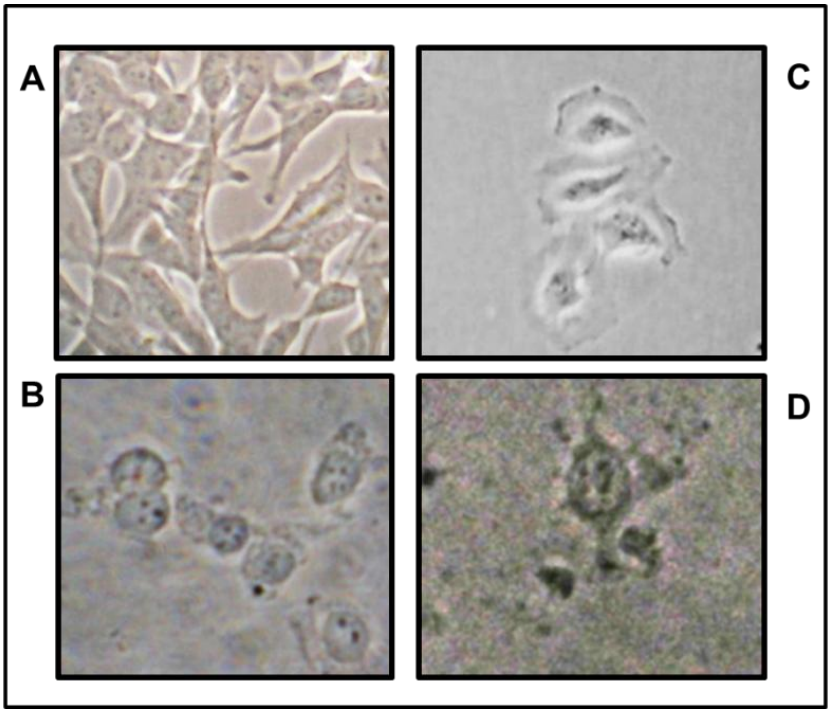

Figure 20: *SH-SY5Y* (A) and *HeLa* cells (C), optical microscope, 10 X; nuclei of *SH-SY5Y* cells (B) and nuclei of *HeL*a cells (D), optical microscope, 40 X.

The samples were used immediately for Western blotting experiments.

SDS-PAGE AND WESTERN BLOTTING ANALYSIS: All samples were diluted in a ratio of 3:1 with sample buffer and they were denatured at 95 °C for 5 minutes. Then the samples were carried, in equal volume, in polyacrylamide 12 % gel and they were underwent to SDS-PAGE.

Various primary antibodies were used for Western Blotting: the *SMN* antibody (purified mouse Anti-SMN, BD Transduction Laboratories™); the *FUS* antibody (FUS monoclonal antibody, Proteintech Group) and the *TDP-43* antibody (TARDBP polyclonal antibody, Proteintech Group). Conversely, the secondary antibodies used are: anti mouse and anti rabbit, both conjugated to horseradish peroxidase (Sigma-Aldrich) .

Finally, the membranes were exposed to ECL (Thermo Scientific), the data capture was done to Imaging system ChemiDoc-it 410 from UVP (BioRad) and the images were analyzed with VisionWorks ® Life Science Software .

Results and discussion.

We did subcellular fractionation to study the protein isoforms and to understand in which cellular fraction these are.

In particular, 34 samples leukocytes were fractionated, including 16 neurological controls, 2 healthy controls and 16 ALS subjects.

Initially, leukocytes of neurological controls were analyzed to understand how *SMN* protein is expressed when disease is not present.

The experiment was repeated three times and the result was always the same: different *SMN* isoforms. All isoforms are both in the total cells lysed and in the nuclear fraction but the cytoplasmic fraction has not the smaller isoform. Furthermore the larger *SMN* isoforms (40 kDa) seems to have a higher level of expression in the nuclear fraction (figure 21).

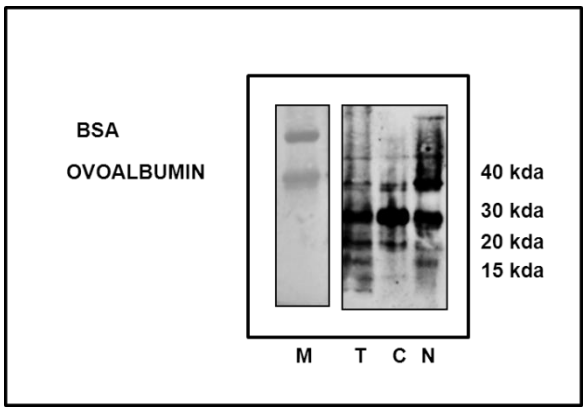

Figure 21: In the first lane the molecular weight markers was loaded (M); in the second lane western blotting for SMN from whole leukocytes of a neurological control (T), third lane western blotting cytoplasmic fraction of the same subject (C) and in the fourth lane nuclear fraction (N). Purified mouse Anti-SMN (BD Transduction Laboratories™1:5000); anti mouse 1: 10000.

Later, ALS leukocytes were analyzed but this experiment was done only one time. The result, that does not appear, shows the absence of the higher molecular weight *SMN* isoform. The data need to be confirmed but they suggest that a isoform of *SMN* protein is lost in patients with ALS. If this result will be confirmed, we can say that *SMN* gene is not compromised in ALS but the *SMN* protein is modified in this disease.

The same experiments were done to study *TDP-43* isoform. From the figure 22 it is possible to see that multiple *TDP-43* isoforms are in leukocytes of neurological

controls but the one with higher molecular weight is found only in the nucleus. These experiments were performed twice and each time the result was the same.

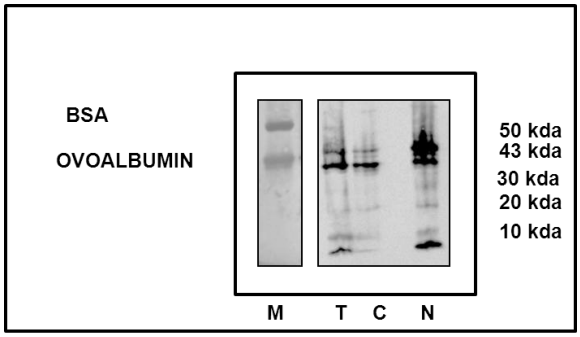

Figure 22: In the first lane molecular weight markers was loaded (M); in the second lane western blotting for *TDP-43* from whole leukocytes of a neurological control (T), third lane western blotting cytoplasmic fraction of the same subject (C) and in the fourth lane nuclear fraction (N). TARDBP polyclonal antibody (Proteintech Group 1:10000); anti rabbit 1:10000.

The experiment in leukocytes of ALS subjects was done only one time and the result seems to be the opposite of the result obtained from the control subjects. In fact, in the nucleus only the 43 kDa isoform appears and it seems that there is a smaller amount of protein in nucleus than cytoplasm (figure 23).

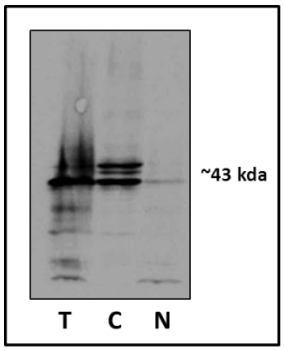

Figure 23: In the first lane western blotting for *TDP-43* from whole leukocytes of a ALS subjects (T), second lane western blotting cytoplasmic fraction of the same subject (C) and in the third lane nuclear fraction (N). TARDBP polyclonal antibody, Proteintech Group 1:10000; anti rabbit 1:10000.

This element must be confirmed but it recalls what is reported in the literature. Namely, that when *TDP-43* is mutated, it translocates to the cytoplasm. Conversely, we analyzed ALS patients who do not have this mutation. Then if these data will be confirmed, we can say that the *TDP-43* translocates to the cytoplasm even if mutation is not present.

Finally, the same experiments were done for *FUS* protein. Many isoforms were seen also here. In particular, an isoform of *68* kDa is in the nucleus and it is not present in the cytoplasm (figure 24).

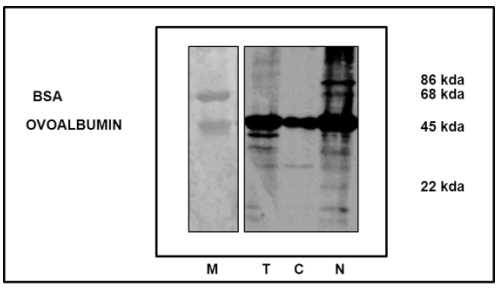

Figure 24: In the first lane was loaded molecular weight markers (M); in the second lane western blotting for *FUS* from whole leukocytes of a neurological control (T), third lane western blotting cytoplasmic fraction of the same subject (C) and in the fourth lane nuclear fraction (N). FUS monoclonal antibody (Proteintech Group 1:10000); anti mouse 1:10000.

These three proteins have been also studied in human cell cultures (*HeLa* and *SH-SY5Y* cells) to have the models on which to do further analysis to understand more about their pathways.

Multiple isoforms of each protein studied are in these cells.

In detail, an only *SMN* isoform (45 kDa) is in *SH-SY5Y* cells. Conversely, many isoforms of *FUS* and *TDP-43* proteins are in the same cells and they are mainly present in the nucleus (figure 25).

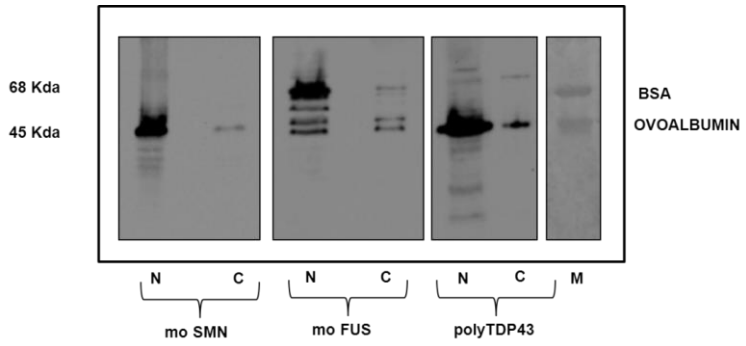

Figure 25: In the first lane western blotting for SMN from nuclear fraction of *SH- SY5Y* cells and the in second lane there is the cytoplasmic fraction of the same cells, third lane western blot for FUS from nuclear fraction of *SH-SY5Y* cells and in the fourth lane there is cytoplasmic fraction of the same cells, in the fifth lane western blotting for *TDP-43* from the nuclear fraction of *SH- SY5Y* cells and in sixth lane there is cytoplasmic fraction of the same cells , in the lastly lane there is molecular weight marker. Purified mouse Anti-SMN (BD Transduction Laboratories™1:5000), anti mouse 1: 10000; FUS monoclonal antibody (Proteintech Group 1:10000), anti mouse 1:10000; TARDBP polyclonal antibody (Proteintech Group 1:10000), anti rabbit 1:10000.

In *HeLa* cells, the *SMN* protein is present in a single isoform about 44 kDa both in the nucleus and in the cytoplasm (figure 26). In the same cells, the *FUS* protein is the same both in the nucleus and in the cytoplasm but it appears to be higher in the nucleus than in the cytoplasm (figure 26).

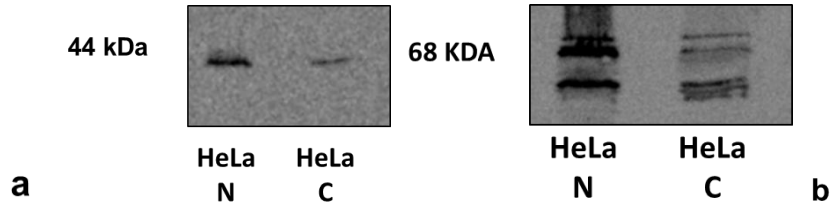

Figure 26: a) In the first lane western blot for *SMN* from nuclear fraction of *HeLa* cells, in the second lane western blot for *SMN* from the cytoplasmic fraction of the same cells. Purified mouse Anti-SMN (BD Transduction Laboratories™1:5000); anti mouse 1: 10000. b) In the first lane western blot for *FUS* from nuclear fraction of *HeLa* cells, in the second lane western blot for *FUS* from the cytoplasmic fraction of the same cells. FUS monoclonal antibody (Proteintech Group 1:10000); anti mouse 1:10000.

Conclusions.

We confirm that different *SMN* isoforms are in the various cell types analyzed, human leukocytes , *HeLa* cells and neuroblastoma cells (*SH-SY5Y*).

In fact, in human leukocytes the *SMN* protein is present in 40 kDa, 30 kD, 20 kDa and 15 kDa isoforms; in *HeLa* cells the same protein is only 44 kDa isoform and in *SH-SY5Y* cells the 45 kDa *SMN* isoform is the most expressed.

La Bella et al in 2004, have explained the existence of these isoforms showing that the *SMN* protein undergoes post-translational modifications. Rat *SMN* mRNA does not undergo alternative splicing; on the contrary, the human *SMN* mRNA can undergo this process. This could give another explanation to the existence of different *SMN* isoforms but to assert this RT-PCR experiments must be done.

In addition, we show that different *SMN* isoforms are in the various cell compartments. Especially, the differences are between the nucleus and the cytoplasm of human leukocytes (figure 21), in particular the smaller isoform is absent in the cytoplasm and the larger one seems to be less expressed in the same cell compartment.

Then we analyzed the *TDP-43* expression in human leukocytes and neuroblastoma cells (*SH-SY5Y*). Multiple *TDP-43* isoforms are in leukocytes of

neurological controls (figure 22) and the isoform with higher molecular weight was found only in the nucleus.

Conversely, the experiment in ALS leukocytes (figure 23) shown that only the 43 kDa *TDP- 43* isoform is in the nucleus of these cells and a less amount of it is present in the nucleus than in the cytoplasm. The last data must be confirmed but if these data will be confirmed.

In neuroblastoma cells (figure 25), multiple isoforms of *TDP-43* appear and both in the nucleus and in the cytoplasm the 43 kDa *TDP- 43* isoform is the most expressed.

Finally, we have studied the *FUS* expression in human leukocytes, in neuroblastoma (*SH-SY5Y*) and in *HeLa* cells. In human leukocytes (figure 24) the *FUS* protein is present in different isoforms and in particular the *68 kDa* specific isoform was found in the nucleus. However difference is not present between control and ALS patients analyzed. The same results were found in *HeLa* (figure 26) and in *SH-SY5Y* cells (figure 25).

Therefore, *SMN*, *TDP-43* and *FUS* proteins have various isoforms that are often found in different cellular compartments. Probably, as they are multifunctional proteins, each isoform corresponds to a different protein function.

In addition, in case of ALS (figure 23 and other data not shown) some *SMN* and *TDP43* isoforms seem to be lacking or moving into the cytoplasm. These data must be confirmed but suggest that although in the pathogenesis of ALS the mutations are not in the DNA, an involvement of the studied molecules may be at the protein level.

For this reason, in the future we will be studied the meaning of these isoforms, paying attention to the post-translational modifications, through specific assays, and analyzing alternative splicing, through RT-PCR studies.

Experiments on leukocytes ALS will be also repeated several times to understand whether a difference in the expression of isoforms is present between controls and diseased patients and if this difference may contribute to the pathogenesis of ALS .

CHAPTER-3

CHAPTER 3- *FUS*, *TDP43* AND *SMN* PROTEINS LOCALITAZION IN *HeLa* AND *SH-SY5Y* CELLS.

Introduction and objectives.

After studying *SMN*, *TDP-43* and *FUS* protein isoforms and after showing that they often have a different distribution in the cells, the analysis was continued by immunofluorescence. We done this to study their subcellular localization in human cell lines: *HeLa* and in neuroblastoma cells (*SH-SY5Y*).

Already in 1996, *Liu Q and Dreyfuss G*. shown that the *SMN* protein was expressed in not only *Gems* of *HeLa* cells but also in the cytoplasm (107).

It has also been seen that the inhibition of the *SMN* expression by RNA interference leads to defects in *Cajal body* formation in *HeLa* cells (108).

The *Cajal bodies* (*CBs*) and the *Gems* (gemini of Cajal bodies) are nuclear structures that are not bounded by a membrane and in many cell types the two structures co-localize (109).

The *CBs* are generally found in nucleoplasm but they have been detected in nucleolus of human breast carcinoma cells and of brown adipocytes. A specific *CBs* marker is p80-coilin protein (109).

The *CBs* are dynamic structures; in fact they disassemble during mitosis and they reassemble in G1 phase of cell cycle (110).

The *snRNPs* (small nuclear ribonucleoproteins) move first through the *Cajal bodies*, then pass to the *speakles* and finally reach the nucleolus (111) (112).

In the nucleus, the *SMN* has been proposed to have a direct role in *pre-mRNA* splicing, possibly involving recycling of splicing complexes (113).

The *Gems* are the nuclear site of *SMN* accumulation. In one study it was observed that these bodies are extremely rare in undifferentiated cells but they are more readily detected upon differentiation (114).

In another study, it was found that the simultaneous non-inclusion of exon 5 and exon 7 in *SMN* leads to the decrease in the number of *Gems* and that the amino-terminal domain of *SMN* is also essential for the correct cellular distribution (115).

In 2008, it was demonstrated that *TDP-43* protein continuously shuttles between the nucleus and the cytoplasm, in a transcription-dependent manner. Specifically, it was shown that C-terminus portion of this is essential for cellular localization, in fact the disruption of the RNA-recognition domain causes the decrease of *TDP-43* protein in the nucleoplasm. Then, its C-terminus portion and its RRM1 domain are essential for correct cellular localization. If mutated, this protein accumulates in stress granules (116) (117).

In ALS, *Gems* are lost and *Hitomi Tsuiji et al*. found that *TDP-43* is located in these bodies of many cells (118). Furthermore, in a recent study it was found that *TDP-43* controls the distribution of *SMN* in *Gems* of motor neurons of *TDP43* transgenic mice (119).

The FUS protein is also mis-localized in ALS (117). In fact, FUS is localized mainly in the nucleus but mutations in the C-terminus of FUS show neuronal cytoplasmic *FUS*-positive inclusions, because a signal which is needed for nuclear import is in this domain (120).

Materials and methods.

CELL CULTURE: As shown in chapter 2.

IMMUNOFLUORESCENCE: The *HeLa* cells and *SH-SY5Y* cells are grown on cover slips in 4-well plate, washed with cold D-PBS Dulbecco's Phosphate Buffered Saline (Euroclone), fixed for 30 minutes with 4% paraformaldehyde at room temperature, washed 3 times with cold D-PBS Dulbecco's Phosphate Buffered Saline (Euroclone), treated for 15 minutes with 50 mM ammonium chloride (NH_4Cl) at room temperature to decrease the autofluorescence of paraformaldehyde, permeabilized with PBS TRITON 0.5% for 10 minutes at room temperature and incubated for one hour at room temperature with 2% BSA to block nonspecific sites. Later specific primary antibodies were added and they were kept overnight at 4 °C, the specific primary

antibodies used are: *SMN* antibody (purified mouse Anti-SMN, BD Transduction Laboratories™); *FUS* antibody (FUS polyclonal antibody, Proteintech Group); *TDP-43* antibody (TARDBP polyclonal antibody, Proteintech Group).

The following day, after the primary antibodies were removed from the wells, three washes with PBS 0.1% TRITON were done, then specific secondary antibodies were added and they were incubated for 1 hour and 30 minutes, in the dark, at room temperature.

In particular, the secondary antibodies used are an anti-mouse antibody conjugated to FITC (Fluor Chemicon) for recognizing the anti-*SMN* and an anti-rabbit conjugated to Cy3 (Cy3 Chemicon) for recognizing the anti-*FUS* and anti *TDP-43*. FITC fluorochrome excites to a wavelength of 450 nm (blue) and emits a light of wavelength of 532 nm (green) and Cy3 fluorochrome excites at a wavelength of 532 nm (green) and emits at 650 nm (red).

Subsequently, the cover slips were placed on a glass slide using gel mount (Sigma), and frozen at -20 C.

For the analysis of the experiments a confocal laser scanning microscopy (Olympus FV-300 equipped with argon, 488 nm and helium/neon, 543 nm, lasers) or Olympus IX70 fluorescence microscope were used.

When we used the fluorescence microscope we used the Image J program to make overlapping images.

Results and discussion.

In our experiments, in *HeLa* cells (figure 27), *SMN* protein is localized in the cytoplasm, in *Gems* and in the nucleoplasm; the *FUS* protein is only located in the nucleoplasm.

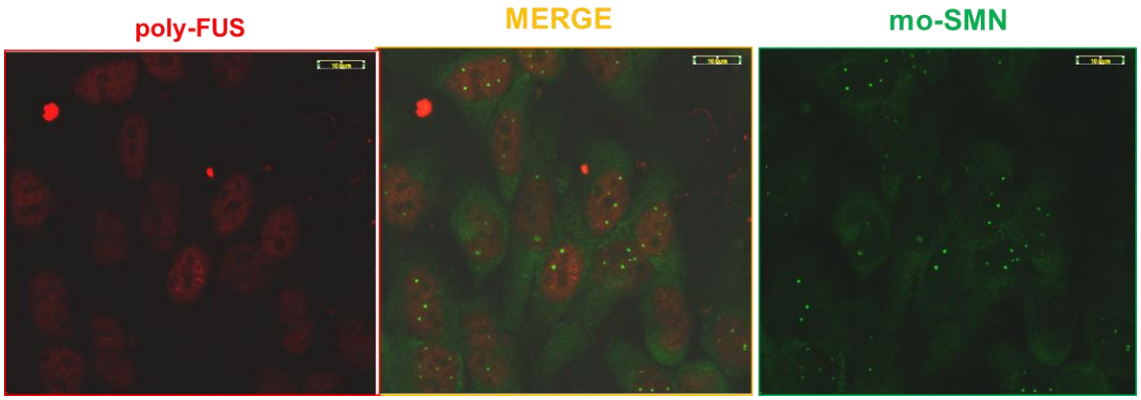

Figure 27: Confocal microscope image of *HeLa* cells. We used anti-SMN (mouse, 1:150, BD Transduction Laboratories TM) and poly- FUS (rabbit, 1:200, Proteintech Group), then we used secondary anti-mouse FITC conjugated (1:1000) (green) and anti-rabbit Cy3 conjugated (1:2000) (red).

In *SH-SY5Y* cells (figure 28) *SMN* and *FUS* proteins have the same distribution of *HeLa* cells but the *Gems* are less than the *HeLa* cells.

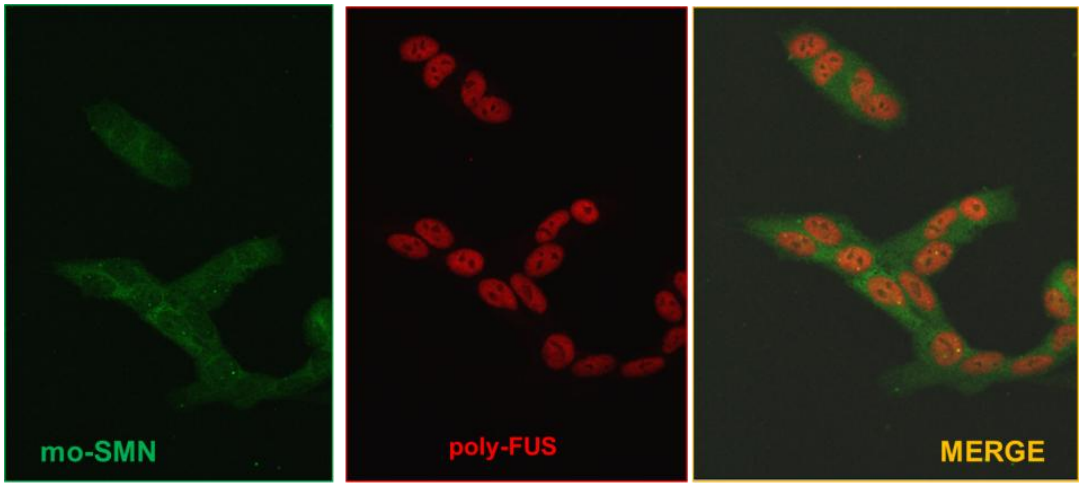

Figure 28: Confocal microscope image of *SH-SY5Y* cells. We used anti-SMN (mouse, 1:150, BD Transduction Laboratories TM) and poly- FUS (rabbit, 1:200, Proteintech Group), then we used secondary anti-mouse FITC conjugated (1:1000) (green) and anti-rabbit Cy3 conjugated (1:2000) (red).

These results were presented in a poster for *SINS Congress*, Rome, October 3 to 5 (*P02-116*).

Overall we show that *FUS* and *SMN* levels are higher in the nucleus in *HeLa* and neuroblastoma *SH-SY5Y* cells. In particular, the *FUS* protein is always diffusely expressed in the nucleoplasm in both cell lines (figure 27 and figure 28). However, the *SMN* protein in *HeLa* cells (figure 27) predominates in *Gems* but also shows a

detectable expression in the cytoplasm and in the nucleoplasm; in *SH-SY5Y* cells, *Gems* are relatively rare (figure 28). These experiments were done several times.

Then we analyzed the *TDP-43* localization and we saw that it is a nuclear and cytoplasmic protein.

In one study it was found that *TDP-43* protein is located in the *Gems* (118) but we found that in the *HeLa* cells, the *TDP-43* protein is not in these sub-nuclear bodies (figure 29).

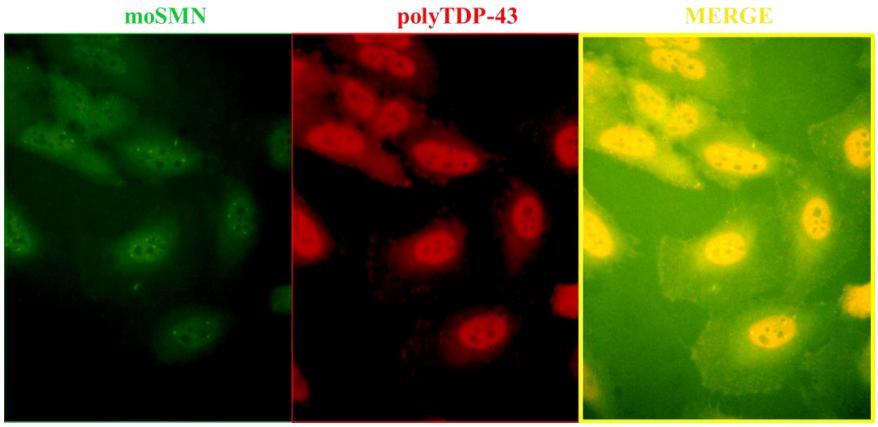

Figure 29: Fluorescence microscopy image of *HeLa* cells. We used anti-SMN (mouse, 1:150, BD Transduction Laboratories TM) and anti-TD-P43 (rabbit, 1:200, Proteintech Group), then we used secondary anti-mouse FITC conjugated (1:1000) (green) and anti-rabbit Cy3 conjugated (1:2000) (red). The overlap of the images was done using the program Image J.

The same result was also obtained in *SH-SY5Y* cells (figure 30); *TDP-43* protein is found both in the nucleus and in the cytoplasm.

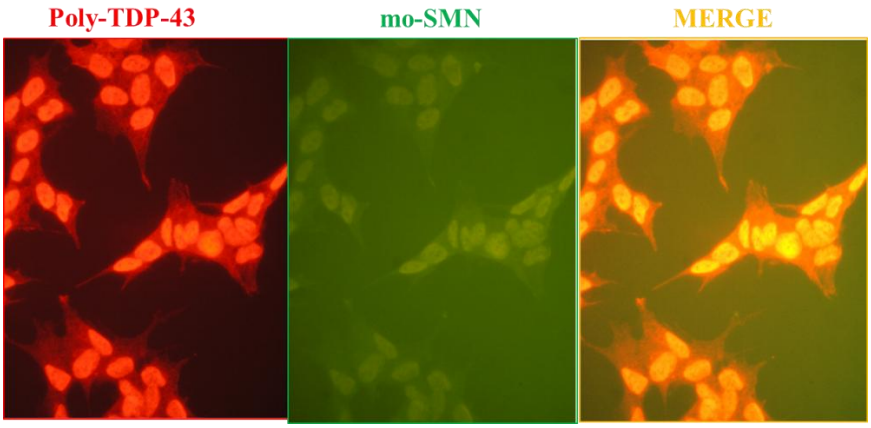

Figure 30: Fluorescence microscopy image of *SH-SY5Y* cells. We used anti-SMN (mouse, 1:150, BD Transduction Laboratories TM) and anti-TD-P43 (rabbit, 1:200, Proteintech Group), then we used secondary anti-mouse FITC conjugated (1:1000) (green) and anti-rabbit Cy3 conjugated (1:2000) (red). The overlap of the images was done using the Image J program.

Therefore, in both cell types *SMN* and *TDP-43* proteins are found in the nucleoplasm and in the cytoplasm. The *FUS* protein is found only in the nucleoplasm.

CHAPTER-4

CHAPTER4- ANALYSIS OF THE SMN PROTEIN INTERACTIONS IN *SH-SY5Y* CELLS.

Introduction and objects.

In immunofluorescence experiments we have seen that *FUS* and *SMN* proteins are in the nucleoplasm, then we decided to test their interaction.

In 2012, this interaction was communicated by *Tomohiro Yamazaki et al.* (121) in *HeLa* cells so we decided to study this in neuroblastoma cells (*SH-SY5Y*).

Materials and methods.

CELL CULTURE: *SH-SY5Y* cells were grown as shown in chapter 2.

SUBCELLULAR FRACTIONATION OF CELLS: as shown in chapter 2.

SMN-IMMUNOPRECIPITATION: Cells were grown to about 80% confluency, then they were washed with cold D-PBS Dulbecco's Phosphate Buffered Saline (Euroclone) and they were mechanically detached from the plate, on ice, with an IP lysis buffer (20 mM TRIS HCl pH 8; 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, protease inhibitors).

The cell suspension was immediately put in gentle rotation, at 4 °C for 30 minutes, then it was centrifuged at 12000 RPM for 20 minutes at 4 °C. The sample was immediately placed on ice and the supernatant was recovered, polyclonal anti-Bcl_2 (Santa Cruz Biotec, Santa Cruz, CA) was added to it and it was incubated for 1 hour, at 4 °C, in gentle rotation.

Subsequently, sepharose beads linked to protein G (Protein G Sepharose 4 Fast Flow, Amersham Pharmacia Biotec AB) were added to it and it was incubated for 30 minutes, at 4 °C, in gentle rotation. This was done to mask non specific sites.

Immediately, the samples were centrifuged for 10 minutes, at 12000 RPM, at 4 °C, the supernatant was recovered and antibody anti *SMN* (0.4 µg / mL) was added and it was incubated overnight at 4 °C, in gentle rotation.

The next day, sepharose beads linked to protein G (Protein G Sepharose 4 Fast Flow, Amersham Pharmacia Biotec AB) were added to the sample and an incubation was done for 4 hours, at 4 °C, in gentle rotation.

Then the samples were centrifuged for 3 minutes, at 2500 RPM, at 4 °C; the pellet was recovered and washed three times, using an IP buffer each time and centrifuged at 5000 RPM, for 5 minutes, at 4 °C.

Once the last supernatant was removed, the sample buffer was added to samples and they were incubated at 95 °C, for 5 minutes and then were used for Western Blotting.

SDS-PAGE AND WESTERN BLOTTING ANALYSIS: As shown in chapter 2 but the samples were carried in polyacrylamide 15 % gel.

For Western Blotting the primary antibodies used were: the SMN antibody (purified mouse Anti-SMN, BD Transduction Laboratories™) and the FUS antibody (FUS monoclonal antibody, Proteintech Group). The secondary antibodies used were anti mouse conjugated to horseradish peroxidase.

Results and discussion.

To understand whether the proteins *FUS* and *SMN* interact in *SH-SY5Y* cells we did *SMN-FUS* co-immunoprecipitation experiments in triplicate.

It is possible to see the result in figure 31 which shows that the two proteins interact and specifically the *SMN* protein interacts with the 68 kDa *FUS* isoform.

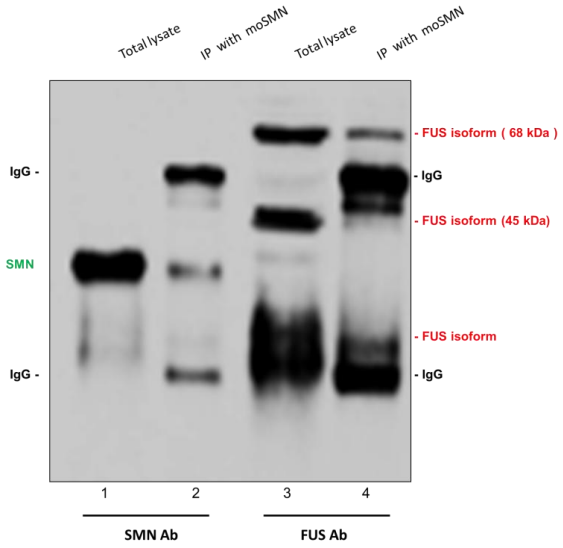

Figure 31: Co-immunoprecipitation *SMN-FUS* from *SH-SY5Y* cells . We used the SMN antibody (BD Transduction Laboratories ^{TM}) to pull down the proteins (IP). The proteins were loaded on an 15% polyacrylamide gel and electrophoresed. After the proteins fall down with the SMN antibody, we used the SMN antibody again for the blot in lane 2 and we used the FUS antibody (Proteintech Group) for the blot in lane 4. In lane 4 both bands correspond to different isoforms of FUS, including the smallest that could be a product of proteolysis of the intact protein, and the bands correspond to the IgG. The isoform of FUS (68 kDa) that interacts with SMN can also be seen. In lane 2 there is a specific band of SMN and there are also the bands that correspond to the IgG. In lane 1 there is SH-SY5Y total cells lysates and we used the SMN antibody for the blot. Conversely in the lane 3 we used the FUS antibody.

These results were presented in a poster at *SINS Congress*, Rome, October 3 to 5 (*P02-116*).

From experiments on the isoforms of *FUS* (Chapter 2), it was shown (figure 24) that the *68 kDa* isoform was mainly in the nucleus. In order to figure out which subcellular compartment the interaction *SMN-FUS* occurs, *SH-SY5Y* cells were fractionated and each cell fraction was studied by *SMN-FUS* co-immunoprecipitation. These experiments show that the two proteins interact (figure 31) and they interact in the nucleus (figure 32). In figure 32 a *FUS* band with low molecular weight appears, that is not present in figure 24. This could be interpreted as a *FUS* isoform that is present in case of protein cleavage, for example due to the alteration of the sample during processing.

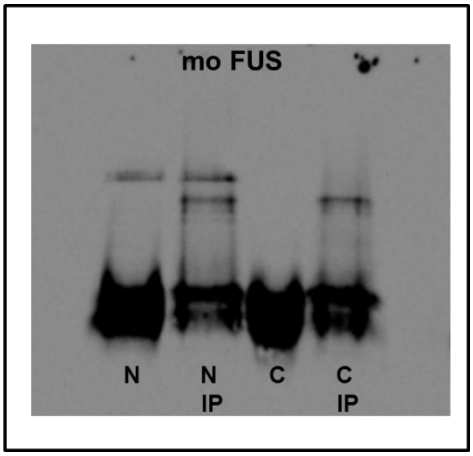

Figure 32: In the first lane: western blot for FUS from nuclear fraction of *SH-SY5Y* cells; in the second lane: same fraction immunoprecipitated with SMN; in third lane: western blot for FUS from the cytoplasmic fraction of *SH SY5Y* cells and in fourth lane: western blot for FUS of the same fraction immunoprecipitated with SMN. The FUS antibody 1:4000 (FUS monoclonal antibody, Proteintech Group), anti mouse 1:10000.

The discovery of this nuclear interaction is very important because both proteins are involved in the pathogenesis of amyotrophic lateral sclerosis.

CHAPTER-5

CHAPTER 5- *SH-SY5Y* DIFFERENTIATION AND *SMN*, *FUS* PROTEINS ANALYSIS.

Introduction and objects.

After discovering that *FUS* and *SMN* proteins are present in *SH-SY5Y* cells in different isoforms and after realizing that these proteins interact in the nucleus of these cells, we wanted to analyze the expression of the same proteins and their interaction during differentiation of neuroblastoma cells (*SH-SY5Y*) in human neuron-like cells.

It was discovered that the *SMN* protein is located in the growth cones and it stimulates neurite outgrowth in cultures (122).

Setola et al. found a particular *SMN* isoform, *a-SMN* (axonal-SMN), which is derived from alternative splicing. This isoform is down-regulated during early development in different tissues. In the spinal cord it is selectively expressed in motor neurons and mainly localized in axons and stimulates motor neuron axonogenesis (123).

In one study, it was shown that the *FUS* expression decreases during differentiation of *SH-SY5Y* cells with retinoic acid but its localization does not change (124).

In Drosophila, abnormalities in the *FUS* protein cause alteration of the neuromuscular junction (125).

The neuroblastoma cell line, *SH-SY5Y*, can be induced to undergo differentiation *in vitro* by addition of retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (126).

Materials and methods.

DIFFERENTIATION: Before plating the cells, the plates were pretreated with poly-ornithine (Sigma-Aldrich, 1x in boric acid) or poly-D-lysine (Sigma-Aldrich,100 µg/ml in bi-distilled water).

Then the *SH-SY5Y* cells were seeded in a multi-well plate and in a 75 cm^2 Falcon flask, in Dulbecco's modified Eagle's medium (DMEM, Euroclone)

supplemented with Nutrient Mixture F-12 (1:1, GIBCO) and with 10% fetal calf serum (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ penicillin and 100 µg streptomycin (Euroclone).

The cells were placed in an incubator for one day and the next day they were looked at under the light microscope. After confirming their adherence to the plate, the cells were treated with retinoic acid (Sigma-Aldrich).

We used different concentrations of retinoic acid (10 µM, 20 µM, 40 µM) but we saw that changing the concentration has not effect on the final result.

We carried out several cycles of treatment with retinoic acid to test the changes in protein localization. About every two days the medium was changed and retinoic acid was added again.

IMMUNOFLUORESCENCE: As described in Chapter 3.

Results and discussion.

Overall, the experiments of neuroblastoma cells differentiation into neuronal-like cells did not show any change in the cellular distribution of the *FUS* and *SMN* proteins. Specifically, we had the best level of differentiation in the following treatment with retinoic acid for 15 days (figure 33).

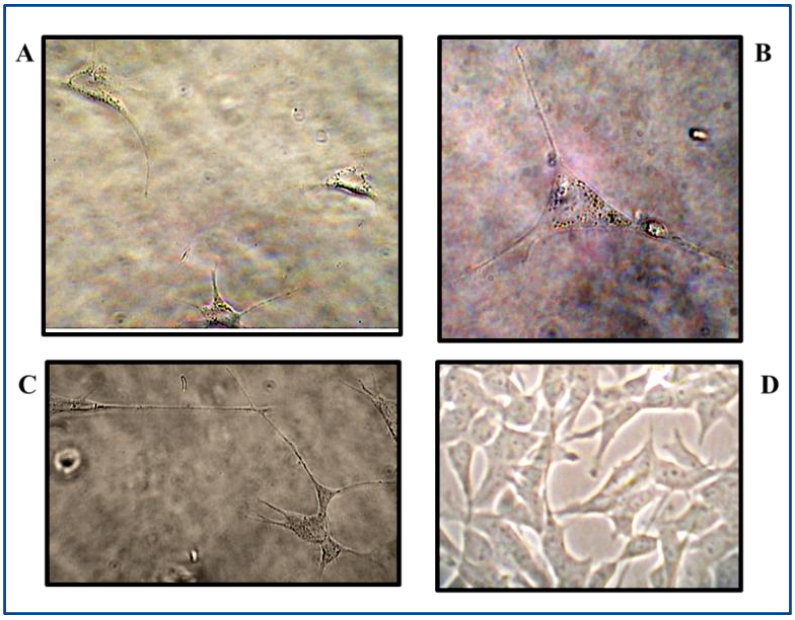

Figure 33: Optical microscope images; in images A, B, C, *SH-SY5Y* cells after about 15 days of treatment with retinoic acid; in D *SH-SY5Y* undifferentiated cells.

During the various differentiation experiments it was noted that the deficiency of nutrients (culture medium not changed) and the presence of a low number of cells stimulate differentiation. During differentiation there is also a loss of cells in the initial phases and then the proliferation slows down. In fact, very few cells are in the final stages of differentiation. We noticed that both pretreatments (poly-ornithine or poly-D-lysine) gave the same result but pretreatment with poly-ornithine induces greater cell loss in the early stages.

Overall, in the final stages of differentiation, after about 20 days, almost all cells have neuronal-like morphology and more than 30% of the cells are connected through neuronal network-like.

The experiments were repeated many times.

To study the differentiation, we used an antibody anti-neurofilament 200, which recognizes *intermediate neurofilaments*, that are specific components of the neurons cytoskeleton. They give support to the cells in both the cytoplasm and the nucleus.

In undifferentiated *SH-SY5Y* cells, the *FUS* protein is located in the nucleoplasm and the *SMN* protein is both in the nucleoplasm and cytoplasm (view chapter 3). The same result is maintained in the early stages of differentiation.

During differentiation, the *SMN* protein distributes itself in the neurite growth cone, in perinuclear vesicles and in the axon extension (figure 34). In the figure 35 the *SMN* distribution in axon-like extensions can be seen well .

The *FUS* protein remains highly concentrated in the nucleus but in the advanced stages of differentiation it moves into the cytoplasm and neuritis (figure 34).

By immunofluorescence image (figure 34) we can see that *FUS* and *SMN* proteins perhaps interact in the nucleus. We also performed a co-immunoprecipitation experiment, but we do not present this figure, which showed that the two proteins interact. It will be a future goal to repeat this study to validate the result.

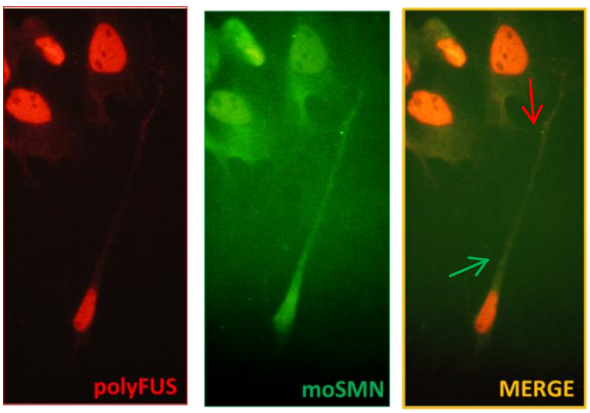

Figure 34: Fluorescence microscope images of *SH-SY5Y* cells during differentiation. In the first picture, from left, cells exposed to antibody purified mouse Anti-SMN, BD Transduction Laboratories™(1:150) and an anti-mouse antibody conjugated to FITC (1:1000); in the second image cells exposed to FUS polyclonal antibody, Proteintech Group (1:200) and an anti-rabbit antibody conjugated to Cy3 (1:2000); in the third image merge of the two images, made with image J program.

In these experiments, we also studied the *intermediate neurofilaments*. They are found both in the cytoplasm and in the axon and they co-localize with the *SMN* protein (figure 35).

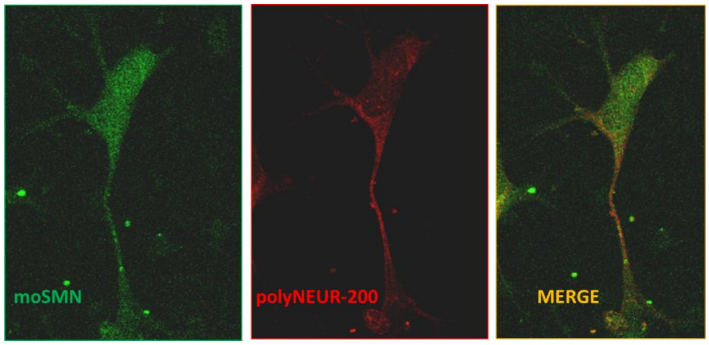

Figure 35: Confocal microscope image, *SH-SY5Y* cells partially differentiated with retinoic acid. In the first picture, from left, cells exposed to antibody purified mouse Anti-SMN, BD Transduction Laboratories™(1:150) and an anti-mouse antibody conjugated to FITC (1:1000), in the second image cells exposed to Neurofilament 200 antibody 1:200 (Anti-Neurofilament 200 polyclonal antibody, Sigma-Aldrich) and an anti-rabbit antibody conjugated to Cy3 (1:2000).); in the third image merge of the two images, made with image J program.

CHAPTER-6

CHAPTER 6- *SMN* AND *FUS* PROTEINS ANALYSIS IN HUMAN FIBROBLAST OF AN ASYMPTOMATIC SUBJECT WITH THE *P525L* MUTATION IN THE *FUS* GENE.

Introduction and objects.

FUS mutations are responsible for ~3% of familial amyotrophic lateral sclerosis and for < 1% of sporadic ALS (128).

The *P525L* mutation was found in a Sicilian patient but also in two Japanese sisters who had a very aggressive and rapid disease progression (129) (130). In addition, the same mutation was found in a 11 year old girl with juvenile sporadic amyotrophic lateral sclerosis (131). So this mutation is present in both sporadic and familial ALS and it is characterized by a very aggressive phenotype.

In a recent study, *Sabatelli et al*. showed that the *FUS R521C* mutation causes *FUS* cytoplasmic translocation (132).

In another study, done by *D Domann et al*, it has been reported that in many cases of familial amyotrophic lateral sclerosis, many *FUS* mutations fall on its C-terminal portion, in particular on the NLS, which is required for its correct localization. Furthermore, the mutations on three arginine to alanine, which are frequently in familial amyotrophic lateral sclerosis (*R521A, R522A, R524A*) cause, in *HeLa* cells, *FUS* cytoplasmic mis-localization. It has also been shown that different mutations cause different mis-localization degrees but no mutation causes complete *FUS* absence from the nucleus and the *P525L* mutation has the worst effect (133) .

The *de novo* mutation in *FUS P525L* causes ALS with juvenile onset and the course of the disease very accelerated (134).

It was also discovered that expression of different *FUS* mutants (*R521C, R521H, P525L*) in neurons causes axonal defects, because the *SMN* protein is accumulated in *FUS* aggregates and therefore the level of this protein decreases in axons (135).

Finally, *FUS* mutations have an effect on snRNP localization (136).

For all these reasons, we investigated *FUS* and *SMN* proteins in fibroblasts from an *asymptomatic* subject with *P525L FUS* mutation. She is the sister and daughter of the subjects who died of ALS. At the same time, her brother and uncle have been studied too because they are healthy subjects without mutations. A neurological control was also analyzed.

Materials and methods.

SKIN BIOPSY: A biopsy was taken of the subject's arm and immediately placed in cold D-PBS Dulbecco's Phosphate Buffered Saline (Euroclone). Subsequently adipose tissue was removed from biopsy and the piece was divided into several parts. Each of them was placed in a well of a multiwell plate, containing Dulbecco's modified Eagle's medium (DMEM, Euroclone) with 10% calf serum (Euroclone), 2 mM L-glutamine (Euroclone) , 100 U/ penicillin and 100 µg streptomycin (Euroclone).

The plate was placed in incubator and it was left there until the fibroblasts were precipitated. When that happened, the biopsy piece was removed and the fibroblasts were transferred into 75 cm^2 Falcon flask.

CELL CULTURE: The human fibroblasts taken from skin biopsy were grown in 75 cm^2 Falcon flask filled in Dulbecco's modified Eagle's medium (DMEM, Euroclone) with 10% fetal calf serum (Euroclone), 2 mM L-glutamine (Euroclone) , 100 U/ penicillin and 100 µg streptomycin (Euroclone).

IMMUNOFLUORESCENCE: As described in Chapter 3. The specific primary antibodies that we used are: The *SMN* antibody (purified mouse Anti-SMN, BD Transduction Laboratories™) and the *FUS* antibody (FUS polyclonal antibody, Proteintech Group). The secondary antibodies that we used are the anti-mouse antibody conjugated to FITC (Fluor Chemicon) for recognizing the anti-SMN and an the anti-rabbit conjugated to Cy3 (Cy3 Chemicon) for recognizing the anti-FUS.

Results and discussion.

We analyzed the distribution of *FUS* and *SMN* proteins in fibroblasts from a patient with a neurologic disease that does not affect the motor neurons (neurological control), from an *asymptomatic* girl with the *FUS P525L* mutation and from her brother and her uncle (healthy controls). From these studies we found that there is not difference between neurological and healthy controls fibroblasts but there is difference between these fibroblasts and those of the girl with *P525L FUS* mutation.

In fact, in all analyzed fibroblasts, the *FUS* protein was in the nucleus, however in those with *P525L FUS* mutation the *FUS* protein was located in both the nucleus and in the cytoplasm. The distribution of *SMN* protein was the same in all samples.

The result of the neurological control fibroblasts is reported in figure 36.

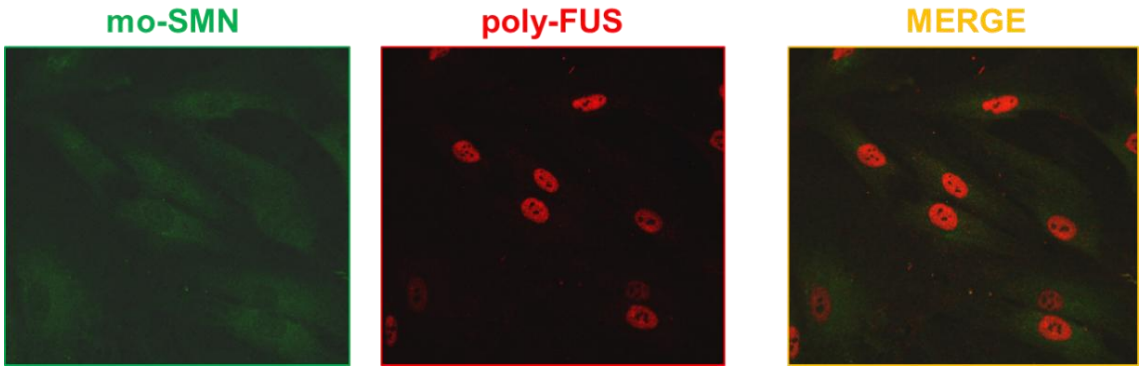

Figure 36: Confocal microscope images of human fibroblast (neurological control). in the first picture, from left, cells exposed to antibody purified mouse Anti-SMN, BD Transduction Laboratories™(1:150) and an anti-mouse antibody conjugated to FITC (1:1000); in the second cells exposed to FUS polyclonal antibody, Proteintech Group (1:200) and an anti-rabbit antibody conjugated to Cy3 (1:2000); in the third image image marge of the two images.

The same result has been obtained from the study of healthy fibroblasts but we do not show the images because they are the same as the neurological control.

In particular in both healthy controls, the *FUS* protein is only in the nucleus in most of the cells analyzed.

Conversely, in subjects with *P525L* mutation, the *FUS* protein is found both in the nucleus and in the cytoplasm in most of the cells analyzed (figure 37).

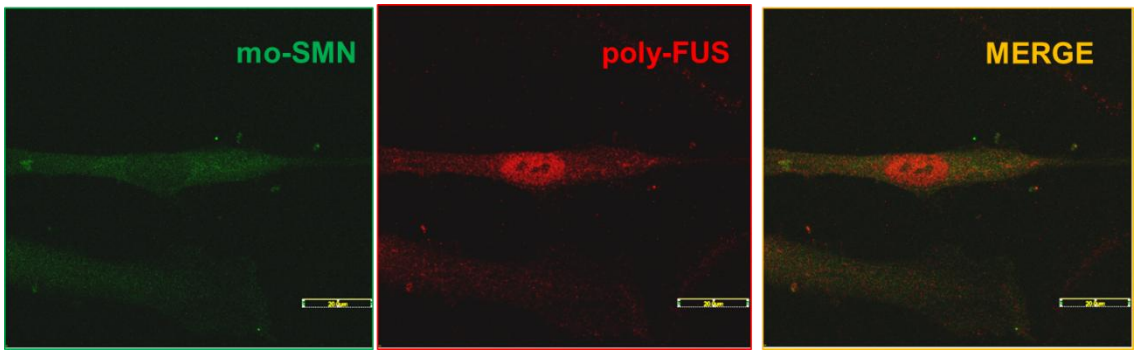

Figure 37: Confocal microscope images of human fibroblast of subjects with P525L mutation in FUS, in the first picture, from left, cells exposed to antibody purified mouse Anti-SMN, BD Transduction Laboratories™(1:150) and an anti-mouse antibody conjugated to FITC (1:1000); in the second image cells exposed to FUS polyclonal antibody, Proteintech Group (1:200) and an anti-rabbit antibody conjugated to Cy3 (1:2000), the image on the right represents the overlap of the other two images.

To validate these findings a statistical analysis was done through the construction of a 2X2 table (table 2) and the implementation of χ^2 test on it.

Table 2 includes the data corresponding to the translocation of the *FUS* protein in the cytoplasm in healthy controls and in subject with the mutation.

OBSERVED 1	CELLS WITH FUS IN NUCLEUS AND IN CYTOPLASM	CELLS WITH FUS ONLY IN NUCLEUS	
MUTATION P525L FUS	88	35	123
CONTROL 1	2	111	113
	90	146	236

Table 2: 2X2 table that includes data corresponding to the analysis of the translocation of the *FUS* protein in the cytoplasm in healthy controls than in subject with the mutation.

The χ^2 tests done on above table returned a χ^2 value of 121,5. The synoptic table of χ^2 shows the critical value of 7.88 at a probability of 0.99. It can be said that, with a 1% probability of error, the frequency of *FUS* translocation is significantly different between the subject with the mutation than in the healthy controls.

Subsequently, these data were reported in a histogram (figure 38), where the error bars represent the absolute error, calculated both for the subject with the mutation (red) and for the healthy controls (blue).

FREQUENCY OF FUS TRANSLOCATED IN MUTATION	FREQUENCY OF FUS TRANSLOCATED WITHOUT MUTATION
0,77	0,03

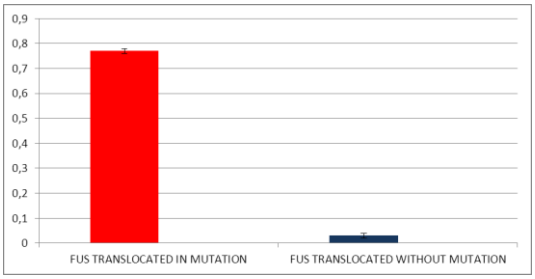

Figure 38: Table and histogram that represent the rate of cells that have *FUS* protein both the cytoplasm and in the nucleus, in subjects with *P525L FUS* mutation that in healthy subjects.

In this graph (figure 38) there is not overlap between the data of the subject with the mutation and control subjects and then the translocation frequencies are different in the two samples. This histogram confirms the data.

Simultaneously, the growth of the fibroblasts of the asymptomatic subject with *P525L FUS* mutation was analyzed.

In particular, the cells were observed for 15 days and it was found that the fibroblasts with mutation *P525L FUS* have a limited growth period.

During the observation, after a couple of days, some vesicles appear in the cells (figure 39) and from this moment on, a decrease in cell growth was noted.

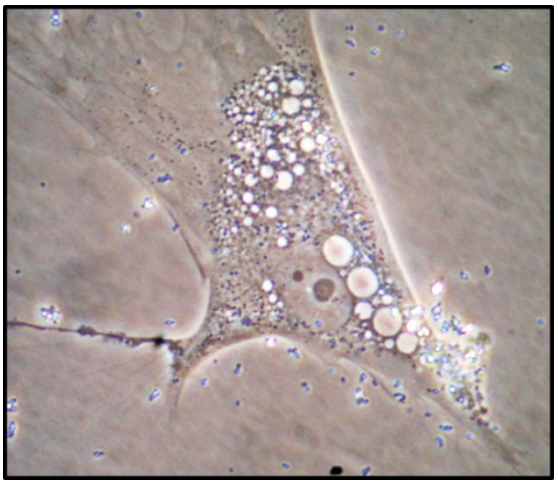

Figure 39: Fibroblast with *P525L FUS* mutation, after a couple of days from its seeding in plate; optical microscope, 40 X.

Immuno-fluorescence experiments were done on these same cells to evaluate the distribution of *SMN* and *FUS* proteins (figure 40).

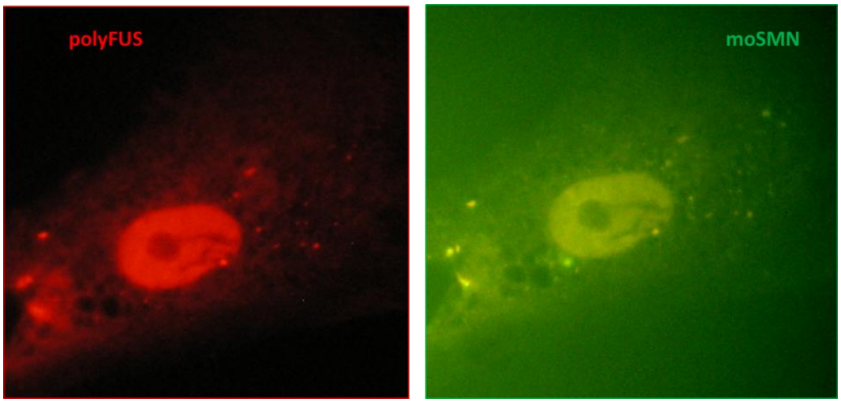

Figure 40: Fluorescence microscope images of human fibroblast of subject with *P525L FUS*, in the first picture, from left, cells exposed to FUS polyclonal antibody, Proteintech Group (1:200) and an anti-rabbit antibody conjugated to Cy3 (1:2000); in the second image, cells exposed to antibody purified mouse Anti-SMN, BD Transduction Laboratories™(1:150) and an anti-mouse antibody conjugated to FITC (1:1000).

Even in these mutated cells, such as those analyzed previously, *FUS* and *SMN* proteins are found both in the nucleus and in the cytoplasm. This demonstrates that their distribution does not change during cell growth.

The two proteins are not in the vesicles but some granules are labeled with both anti-SMN and anti-FUS antibodies.

Therefore, these studies have shown, for the first time, that in case of the *P525L FUS* mutation, even if the subject does not manifest the disease, changes begin in the cells and specifically the *FUS* protein translocates to the cytoplasm, assuming a different location compared to controls, however the distribution of the *SMN* protein remains unchanged.

Even if there is a mis-localization of the *FUS* protein in fibroblasts, yet there is not a manifestation of amyotrophic lateral sclerosis, then in the future, monitoring studies of the *FUS* protein in this subject will be done.

In addition, we will study the specific isoforms involved in this translocation and the *FUS*/*SMN* interaction, to see if there are any changes due to mutation in *FUS* or its mis-localization.

FINALLY CONCLUSIONS.

The amyotrophic lateral sclerosis (ALS) pathogenesis is not yet completely clear. So the PhD research aim was to provide a further contribution to the knowledge on the ALS molecular basis, with particular attention to the role of *SMN*.

Specifically, we studied the *SMN* gene with RFLP-PCR and all our experiments produced the same results:

- from analysis of *SMN* exon 7: in each positive control (SMA patient) a single band always appeared (176 bp), corresponding to the *SMN2* gene, however in each neurological control and in ALS patients two bands always appeared and they corresponded to the *SMN1* (188 bp) and *SMN2* (176 bp) genes;
- from analysis of *SMN* exon 8: in the positive control (SMA patient) two bands always appeared (122 bp and 78 bp), which corresponded to the *SMN2* gene, however in the neurological controls and in ALS patients, there are always three bands (200 bp; 122 bp and 78 bp) , which correspond to the *SMN1* and *SMN2* genes.

These data indicate that, contrary to what happens in spinal muscle atrophy (SMA), the deletion of the *SMN1* gene is not involved in the pathogenesis of ALS. These data gives a further contribution to the understanding of the molecular basis of the disease studied. In fact, there are still conflicting ideas about the role of the *SMN* gene.

In the future, it would interesting to see if the number of the copies of *SMN* genes varies or if there are variations in their promoter that could alter the transcription.

We also analyzed the role of *SMN* in the pathogenesis of ALS at the protein level. In particular we studied the *SMN* protein isoforms to understand whether specific isoforms are involved in the ALS pathogenesis. We also analyzed the isoforms of the *FUS* and *TDP-43* proteins. These are involved in ALS and they are *SMN* potential interactors.

From these studies we confirm that different *SMN* isoforms are in the various cell types analyzed. In fact, in human leukocytes, the *SMN* protein is present in various isoforms of 40 kDa, 30 kD, 20 kDa and 15 kDa. A 44 kDa isoform is only present in *HeLa* cells. the 45 kDa *SMN* isoform is the most expressed in *SH-SY5Y* cells.

In addition, we show that different *SMN* isoforms are in the various cell compartments, especially between the nucleus and the cytoplasm of human leukocytes. In particular we noted that in the cytoplasm the smaller isoform is absent and the larger one seems to be less expressed.

Between the two cell compartments of the *HeLa* and *SH-SY5Y* cells there is only a difference of expression level, in fact the *SMN* seems to be more expressed in the nuclei. It also seems that, in the ALS samples, the 40 kDa isoform of *SMN* is lost but these data need to be confirmed.

We have also analyzed the *TDP-43* expression in human leukocytes and neuroblastoma cells (*SH-SY5Y*). Multiple *TDP-43* isoforms are in leukocytes of neurological controls and the isoform with higher molecular weight is found only in the nucleus. However, the experiment in ALS leukocytes showed an opposite result to that obtained from control subjects. In fact, only the 43 kDa *TDP- 43* isoform is found in nucleus of these cells and there is a smaller amount of this protein. Therefore, if these data can be replicated, we can say that *TDP-43* translocates to the cytoplasm even when it is not mutated and that this translocation may initiate or contribute to the pathogenic process.

Even multiple isoforms of the protein *TDP-43* are in neuroblastoma cells and the 43 kDa *TDP- 43* isoform is the most expressed both in the nucleus and in the cytoplasm.

In conclusion, we studied the *FUS* expression in human leukocytes, in neuroblastoma (*SH-SY5Y*) and in *HeLa* cells. In human leukocytes the *FUS* protein is present in different isoforms. In particular a 68 kDa specific isoform is present in the nucleus. However there is not difference between control and ALS patients analyzed. The same results were found in *HeLa* and in *SH-SY5Y* cells.

Therefore, the three proteins studied, *SMN*, *TDP-43* and *FUS*, have various isoforms that are often found in different cellular compartments.

Then, in the future, the meaning of these isoforms will be studied. Experiments on ALS leukocytes will be also repeated several times to understand whether there is a difference in the expression of isoforms between controls and diseased patients and if this difference may contribute to the pathogenesis of ALS .

After studying *SMN*, *TDP-43* and *FUS* protein isoforms and after seeing that they often have a different distribution in the cells, the analysis was continued by immunofluorescence.

This investigation was done to study if any changes in the proteins distribution are present in different cell types or in the case of particular conditions, such as differentiation or mutation.

In our experiments, the *SMN* protein is localized in the cytoplasm, in *Gems* and in the nucleoplasm in *HeLa* cells. Conversely, the *FUS* protein is only found in the nucleoplasm. In *SH-SY5Y* cells *SMN* and *FUS* proteins have the same distribution of *HeLa* cells but *Gems* less are than *HeLa* cells.

After we saw that both the *FUS* and the *SMN* proteins are in the nucleoplasm we decided to test their interaction in *SH-SY5Y* cells. These cells were fractionated and each cell fraction was studied by *SMN-FUS* co-immunoprecipitation. These experiments show that the two proteins interact in the nucleus and specifically the *SMN* protein interacts with the 68 kDa *FUS* isoform. This is an important finding because both proteins are involved in ALS.

Later, we analyzed the expression of the same proteins and their interaction during differentiation of neuroblastoma cells (*SH-SY5Y*) in human neuron-like cells. Overall, these experiments showed that there is not change in the cellular distribution of the *FUS* and *SMN* proteins during differentation. It has also been shown that the *intermediate neurofilaments* co-localize with the *SMN* protein. As a final analysis, we evaluated the distribution of *FUS* and *SMN* proteins when *FUS* gene is mutated.

Specifically, we investigated this in fibroblasts from an *asymptomatic* subject with the *P525L FUS* mutation. She is the sister and daughter of subjects who died of ALS.

At the same time, her brother and her uncle have been studied too, they are healthy subjects without mutations. A neurological control was also analyzed.

In all the fibroblasts analyzed, the *FUS* protein was in the nucleus however in those with *P525L* mutation, the *FUS* protein was located in both the nucleus and in the cytoplasm. The distribution of *SMN* protein was the same in all samples. Simultaneously with these studies the growth of the fibroblasts was also analyzed.

From these studies we discovered that the fibroblasts with mutated *FUS* have a limited growth period when compared to controls. In fact, after a couple of days, some vesicles appear in the cells and from this moment on, a noted decrease in cell growth occurred.

We will do another study to better understand this process and to have more information on the topic.

Consequently, from my PhD research we discerned that *SMN* is not involved in the pathogenesis of amyotrophic lateral sclerosis at the genetic level. It does however seem to be involved at the protein level because we observed that in the leukocytes of a patient with ALS the 40-kDa isoform of SMN is missing, but this remains to be confirmed. Furthermore, we found that in neuroblastoma cells *SMN* interacts with another protein involved in ALS, *FUS*, and specifically interacts with the 68 kDa *FUS* isoform in the nucleus.

Finally, for the first time we show that an *asymptomatic* subject with the *P525L* mutation in the *FUS* gene has changes in the cellular distribution of the *FUS* protein even before ALS manifests itself.

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