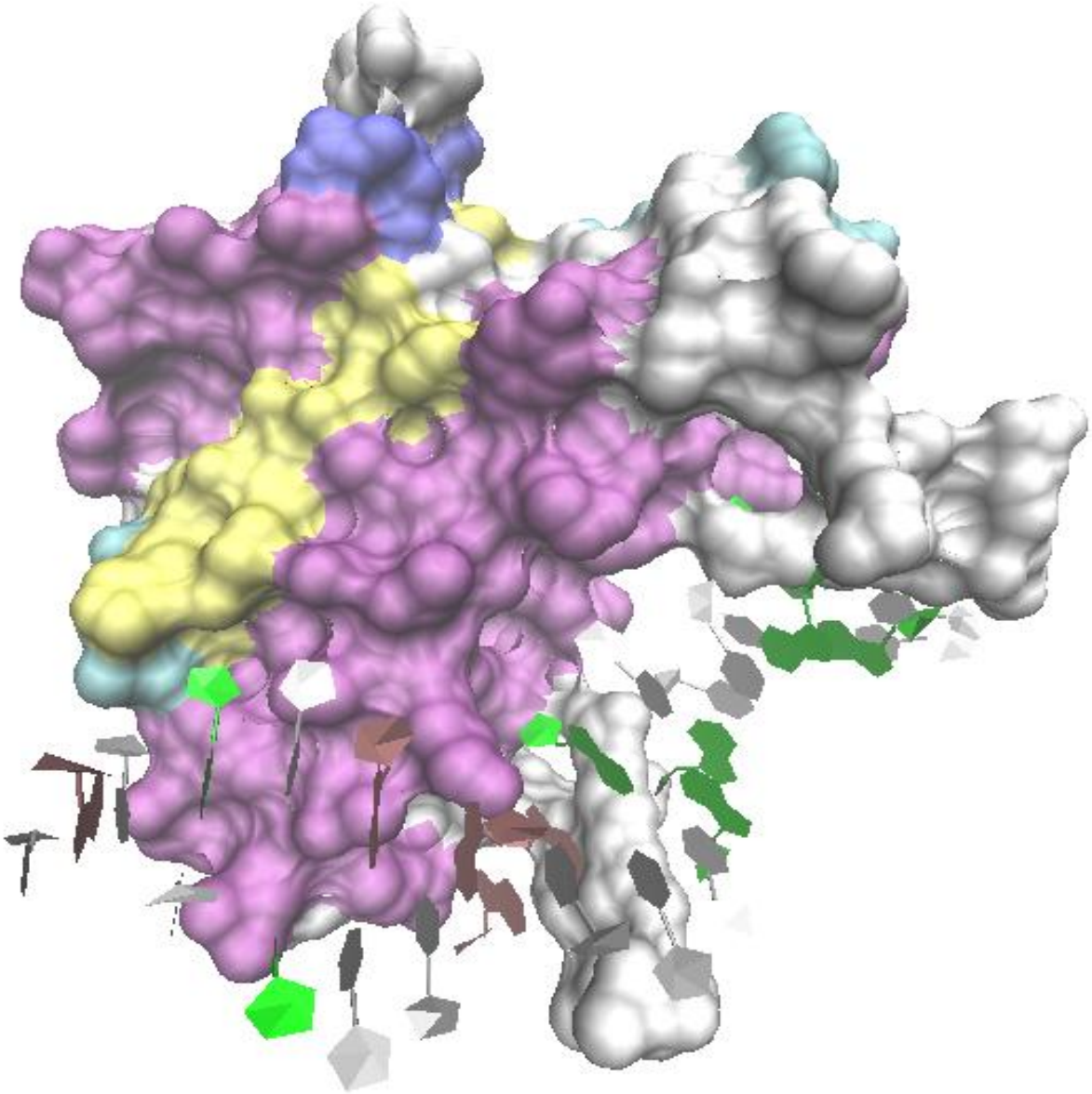
A NOVEL INVOLVEMENT OF SERUM RESPONSE FACTOR IN KENNEDY'S DISEASE



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1. PREFACE

The main focus of this project is Kennedy's disease (KD). This is a rare neurodegenerative disorder a cure against which has not yet been found. So here, in a new approach, I sought to discover the possible involvement of one single protein, known as Serum Response Factor (SRF), in the disease.

To do so, I developed a project at the "IRB Barcelona" embedded inside the "Recerca a Secundària" program for 3 months. This course is lending the opportunity to the students selected from all around Catalonia to develop their own research projects with the aid of a volunteer researcher from the "Parc Científic de Barcelona". It is for that reason that, with the tools that were lent to me in Dr. Salvatella's lab of Molecular Biophyisics and under the supervision of my tutor Bahareh Eftekharzadeh, here I wanted to solve whether or not this protein could be a major hallmark for the neurodegenerative course of this disorder. The importance of the study is based on the fact that if an improper function of this molecule was found there, an explanation of some KD symptoms, the cause of which had not yet been found, could be drawn. Moreover, the unravelling of this protein's key involvement in the disease would even also serve as a source of new therapeutic targets against its degenerative process.

My main aim to develop this project has been the fact that since I was a child I wondered what the underlying mechanisms were behind how could we think, do or act reasonably. Moreover, the processes that are leading to the impairment of this machinery, this is, neurodegenerative diseases, had also been intriguing me since long time gone. It is for that reason that from this point, as I was progressively building my knowledge about this field, I started having a major interest for Kennedy's Disease. The reason for this specially is that this condition can also serve as a model for other fatal

disorders such as Huntington's disease or Spino cerebellar ataxia-3 and so a progress in its understanding could prove of critical importance for future research on these other diseases. For that, I decided to focus my research project on it with the aim of contributing to the long route that is left until the complex puzzle that lies behind its degenerative process can be finally solved.

This project will be structured first in the description of Kennedy's disease, as a general overview, followed then by the central study per. Se. This will consist in an introductory part in which the main proteins' function and their possible relationship with the disease will be explained along with the materials and methods used. Then the main results of the study will be exposed together with the final topics of discussion where possible future approaches will be described. It is the latter the most important part of the project as it will be there where a sense will be given to the results by including them into what had already been done thus serving as a more complete aid in the understanding of the disease.

2. INTRODUCTION TO KENNEDY'S DISEASE

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's Disease (KD), is a type of inherited neurodegenerative disorder characterized by the loss of the lower motor neurons* from the brainstem* and the spinal cord* along with the skeletal muscle cells. Its age onset is situated on average, in between 40 and 50 years old and results in muscle cramps together with a progressive atrophy and weakness. ([1])

The reasons for which this could be happening are extensive but they all depend upon a main hallmark: The increase in the number of Glutamines (Gln)*, an amino acid, in the polyGln (PolyQ) region* from the N-Terminal domain* of the Androgen Receptor (AR). This is consequently leading to the formation of insoluble intranuclear aggregates of this protein when the mutant ARs are bound together through their polyQ tail thus forming a structure that is impairing cellular processes in a wide set of different manners. All this is causing the subsequent death of the cells where these are present. ([2]) However, the steps between the formation of these aggregates and the process of apoptosis* have not yet been drawn completely. One of the main reasons why this could be happening is the recruitment of different proteins inside these fibrils ([3]), some of which could even be crucial for cell maintenance and death inhibition, which, with that, would not be able to perform their function properly thus causing the induction of toxic effects.

The words labelled with a * are further explained at the "Glossary" below.

[1] Poletti, Angelo. "The Polyglutamine Tract of Androgen Receptor: From Functions to Dysfunctions in Motor Neurons."

[2] Fischbeck, Kenneth H., Andrew Lieberman, Christine K. Bailey, Annette Abel, and Diane E. Merry. "Androgen Receptor Mutation in Kennedy's disease."

[3] McCampbell, Alexander, J. Paul Taylor, Addis A. Taye, Jon Robitschek, Mei Li, Jessica Walcott, Diane Merry, et al. "CREB-binding Protein Sequestration by Expanded Polyglutamine."

2.1-THE ANDROGEN RECEPTOR AND TESTOSTERONE

The Androgen Receptor (AR) is the most important protein in Kennedy's disease as its mutation is the most prominent cause of the toxic effects seen in the disorder. For that, it is worth of understanding what its role played is in normal conditions.

Testosterone* is the natural activator of this protein. When this molecule is present in the cytoplasm of the cells, it is converted into dihydrotestosterone (DHT)* by 5α-reductase* which binds afterwards to the AR causing its translocation to the nucleus. However, this can also be achieved through the direct interaction of testosterone. There, this protein further forms a complex with another AR thus assembling a structure known as a dimer* which is then able to activate a wide array of transcription factors, whose function is to induct the activation of the cellular machinery that results in the formation of a new protein.

Despite this fact, AR can also directly act as a transcription factor due to its DNA binding domain which recognizes specific promoter sequences situated at the beginning of a wide set of genes. When bound there, the protein will directly cause their transcription ([4]).

Moreover, it has been pointed out that when there is a mutation in the AR polyQ tail, in KD, there is a nearly complete loss of this protein's function and testosterone is just then able to cause its translocation to the nucleus but not its activation in a process known as androgen insensitivity syndrome (AIS)* ([5]).

[4] Deslypere, J.-P., M. Young, J.D. Wilson, and M.J. McPhaul. "Testosterone and 5α-dihydrotestosterone Interact Differently with the Androgen Receptor to Enhance Transcription of the MMTV-CAT Reporter Gene."

[5] Thomas, Patrick S., Gregory S. Fraley, Vincent Damien, Lillie B. Woodke, Francisco Zapata, Bryce L. Sopher, Stephen R. Plymate, and Albert R. La Spada. "Loss of Endogenous Androgen Receptor Protein Accelerates Motor Neuron Degeneration and Accentuates Androgen Insensitivity in a Mouse Model of X-linked Spinal and Bulbar Muscular Atrophy."

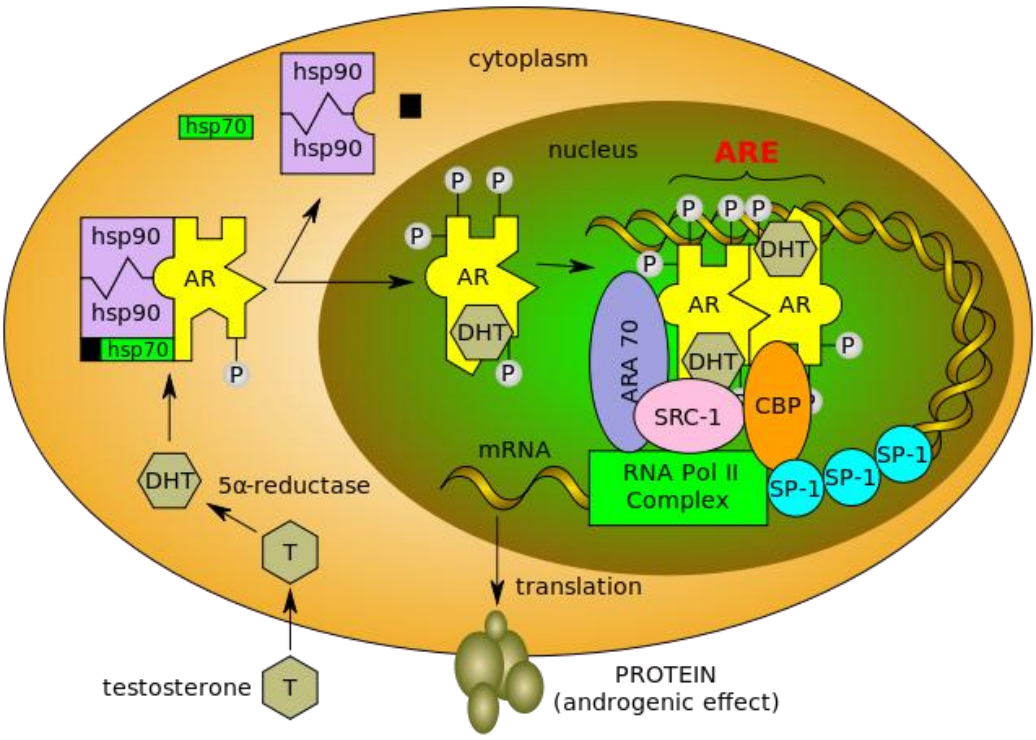


Fig. 1: Schematic representation of the AR activation: As it can be shown here, there is first a conversion of Testosterone into DHT by 5α-reductase which is then causing the nuclear translocation of the AR ([6])

2.2-PATTERN OF INHERITANCE

As mentioned above, KD is an inherited neurodegenerative disease. The cause of the disorder is mainly due to a mutation in the gene codifying for the Androgen Receptor (AR) situated in the X chromosome that is leading to an excessive repetition of the CAG triplet of nucleotides, which is codifying for Glutamine, up to more than 50 to 60 times in the polyQ region of this protein. ([2])

Another important fact related to inheritance is its presence mainly in men because of two reasons:

1. Firstly, this group is more prone to develop it because of the fact that the gene codifying for the protein is just situated in the differential region of the X chromosome, but not on the Y, making possible that the sole presence of the

[6] "Androgen Receptor." *Wikipedia, the Free Encyclopaedia*

mutant gene there, in men, will lead to their expression (See Fig. 2).

2. Testosterone (T) has higher levels in men than in women as it is the main involved in the formation and development of their differential sexual treats and this could lead to an increased rate of degeneration among males due to the abovementioned AIS. However, some mild symptoms have also been detected in females with the mutated allele in both or either one of the chromosomes ([7]).

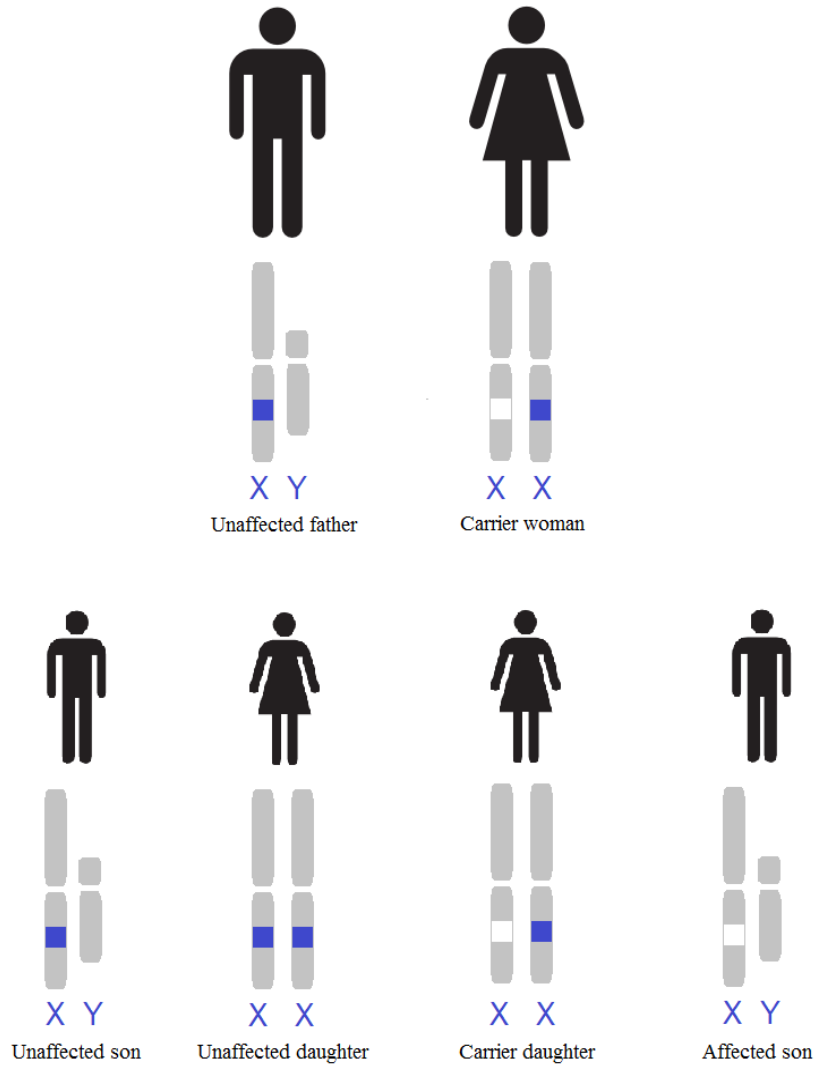


Fig. 2: Schematic representation of the pattern of mutant AR inheritance: As it can be seen, blue stands for the normal AR while white corresponds to the mutant allele (Own elaboration).

[7] Mariotti, Caterina, Barbara Castellotti, Davide Pareyson, Daniela Testa, Marica Eoli, Carlo Antozzi, Vincenzo Silani, et al. "Phenotypic Manifestations Associated with CAG-repeat Expansion in the Androgen Receptor Gene in Male Patients and Heterozygous Females: a Clinical and Molecular Study of 30 Families."

2.3-FIBRILS' FORMATION COURSE

Fibrils' formation is supposedly the most important hallmark in the process of KD degeneration, along with the AR loss of function, due to the fact that key proteins for cell survival would be recruited there thus causing their inhibition. For that reason, it is really worth of understanding the way by which this is happening.

The most widely regarded theory to explain the formation of these structures and its progressive growth is an aberrant sequential interaction between the different ARs through their N-terminal polyQ region, in a conformation known as beta-sheet*, which is causing these proteins to clump together thus leading to the formation of insoluble aggregates.

The steps initiate with the N-terminal domain of these proteins in a partially unfolded state because of the mutation in their polyQ tail which is inducting both a loss of function and an increased tendency of the proteins to aggregate with each other ([8]). This is due to the fact that the conformation in the polyQ-expanded monomers is not favourable in energy terms. For that reason, the different mutant proteins then tend to structure, by binding to each other through their N-terminal domains, into soluble oligomers, which are shorter constructs than the aggregates that are leading, afterwards to the formation of protofibrils through the recruitment of more mutant ARs into these structures. This, with that, accounts for a greater degree of stability. The process is finally ending up with the formation of the fibrils commonly seen in the disease ([9]) (See Fig. 3).

[8] Lyubchenko, Yuri L., Alexey V. Krasnoslobodtsev, and Sorin Luca. "Fibrillogenesis of Huntingtin and Other Glutamine Containing Proteins."

[9] Mark S Forman, John Q. Trojanowski. "Neurodegenerative Diseases: a Decade of Discoveries Paves the Way for Therapeutic Breakthroughs."

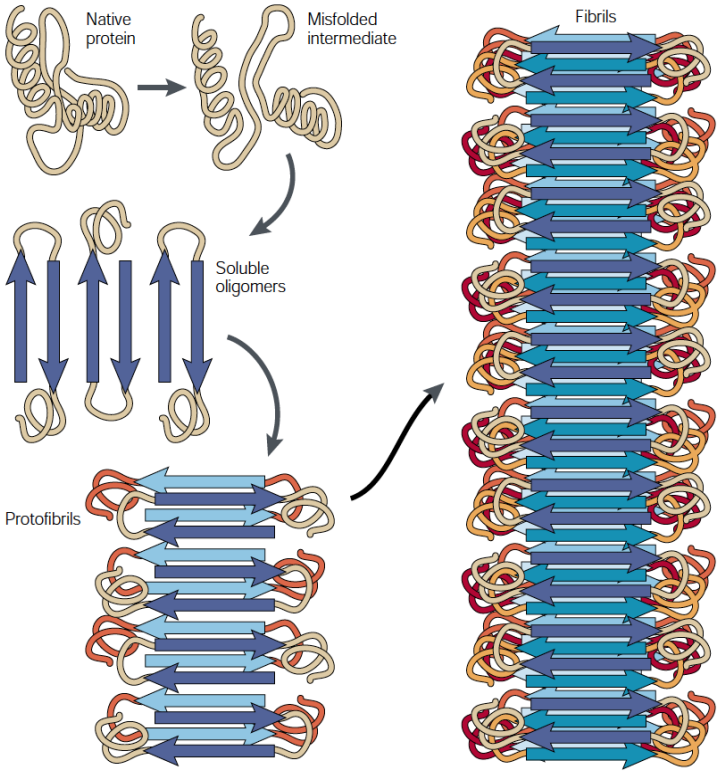


Fig. 3: Schematic representation of the AR fibril formation process. As it can be observed, it all starts with the protein in a partially unfolded state that, by aggregating with others, leads to the formation of soluble oligomers that confer a greater degree of stability. These structures, by progressively growing will end up forming the fibrils seen in KD (Own elaboration).

2.4-OTHER TOXIC EFFECTS

The main target of degeneration in KD had been widely regarded to be the fibrils present inside the nucleus that, as shown above, would be recruiting proteins crucial for the maintenance of cells' survival. However, recent studies point out to other effects of the mutant AR upon the areas affected in KD and that could be as important as the ones exerted by the presence of the aggregates. These are mainly related to the AR loss of function due to the expanded polyQ tail ([10]) because of the fact that this protein is strongly related to the regulation of key genes in cell differentiation and maintenance that, when imbalanced, are not able to fully perform its function thus leading to toxic effects.

[10] Lieberman, Andrew P., George Harmison, Andrew D. Strand, James M. Olson, and Kenneth H. Fischbeck."Altered Transcriptional Regulation in Cells Expressing the Expanded Polyglutamine Androgen Receptor."

Moreover, it has been shown that with the mutant polyQ tail, AR may be disrupting processes in which it had not been regarded to be involved in normal conditions such as the dysregulation of fast axonal transport in motor neurons through the aberrant binding with transport proteins such as kinesins and dyneins*.([11])

To all this it is summed the above mentioned androgen insensitivity that directly correlates with the AR loss of function although its effects and its role played here have not yet been drawn completely. ([12])

[11] "Neuropathogenic Forms of Huntingtin and Androgen Receptor Inhibit Fast Axonal Transport."

[12] Dejager, S., H. Bry-Gauillard, E. Bruckert, B. Eymard, F. Salachas, E. LeGuern, S. Tardieu, R. Chadarevian, P. Giral, and G. Turpin. "A Comprehensive Endocrine Description of Kennedy's Disease Revealing Androgen Insensitivity Linked to CAG Repeat Length."

3. INTRODUCTION TO THE PROJECT DEVELOPED

The aim of this project is mainly focused upon Serum Response Factor, or SRF. This transcription factor is among the most involved in the process of myogenesis, also known as muscle cell formation, along with other effects of major importance in the early development stages of the skeletal muscle ([13]). It has also been regarded to be present in motor neurons where it is repairing axonal injury after a toxic event has occurred ([14]). Apart from this, it is the most important responsible for the reduction in the levels of caspase-3, a protease* which causes the cleavage* of crucial molecules for the cell under apoptotic conditions. It is for that reason that SRF acts as a major regulator of cells' survival ([15]).

It is exerting its effects as a dimer by binding to specific sequences of DNA consisting of $CC(A/T)_6GG$ [Cytosine-Cytosine- $(Adenine/Thymidine)_6$ -Guanine-Guanine] and known as the Serum Response Element (SRE). These are situated at the initial part of multiple genes such as *α-actin*, *c-fos*, *caspase 3* and *HSP70*, which are key regulators of cells' maintenance and apoptosis. Once SRF has bound to the SRE, it is then inducting the activation or inhibition of their transcription.

SRF can be activated through multiple molecular pathways such as MAPK* or RhoA*. However, there are important differences between them in the way by which they are activating this protein. Some of them, such as MAPK are causing the activation through the phosphorylation* of the TCF family of cofactors* which will be further binding to

[13] Li, Shijie, Michael P. Czubryt, John McAnally, Rhonda Bassel-Duby, James A. Richardson, Franziska F. Wiebel, Alfred Nordheim, and Eric N. Olson. "Requirement for Serum Response Factor for Skeletal Muscle Growth and Maturation Revealed by Tissue-specific Gene Deletion in Mice."

[14] Stern, Sina, Daniela Sinske, and Bernd Knöll. "Serum Response Factor Modulates Neuron Survival During Peripheral Axon Injury."

[15] Beck, Henning, Kevin Flynn, Katrin S. Lindenberg, Heinz Schwarz, Frank Bradke, Simone Di Giovanni, and Bernd Knöll. "Serum Response Factor (SRF)-cofilin-actin Signaling Axis Modulates Mitochondrial Dynamics."

SRF and to a specific sequence of DNA close to the SRE to initiate its activity. Others, such as the RhoA pathway induct SRF function through a direct phosphorylation of the protein that causes its dimerization and further activation, although the exact mechanisms by which this is achieved are still unclear. Overall, the different pathways, through its different ways of action can, by this way, induct the transcription of different sets of critical genes all of them regulated by SRF. ([16])

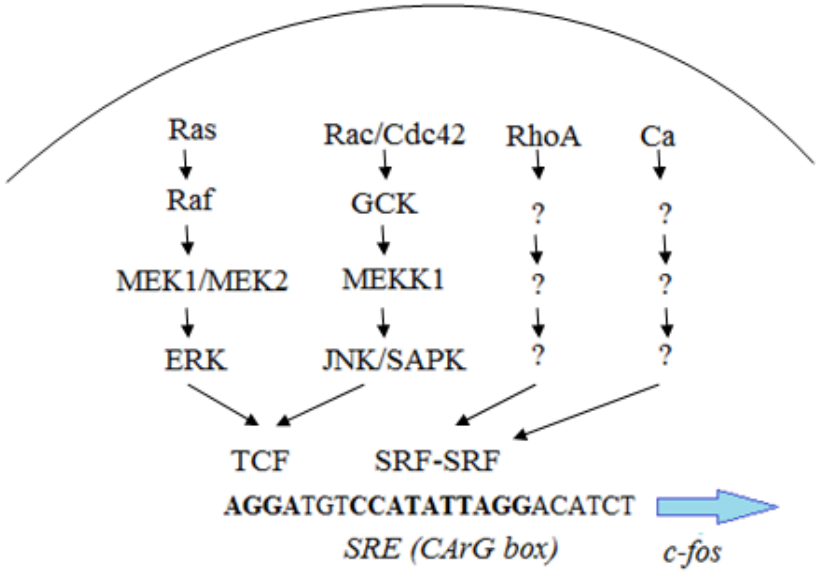


Fig. 4: Set of different pathways that can be inducting SRF activation. In this case the gene transcribed is the one codifying for c-fos (Own elaboration).

However, the most important point of them all about SRF for this project is that AR is inducting the activity of this protein in a critical step for the skeletal muscle development. Moreover, it has already been found that the activation is produced involving multiple regions of both proteins including the ligand-binding domain (LBD)* and, most importantly, the polyQ tail of the AR. ([17])

[16] Chai, J, and A S Tarnawski. "Serum Response Factor: Discovery, Biochemistry, Biological Roles and Implications for Tissue Injury Healing."

[17] Vlahopoulos, Spiros, Warren E. Zimmer, Guido Jenster, Narasimhaswamy S. Belaguli, Steven P. Balk, Albert O. Brinkmann, Rainer B. Lanz, Vassilis C. Zoumpourlis, and Robert J. Schwartz. "Recruitment of the Androgen Receptor via Serum Response Factor Facilitates Expression of a Myogenic Gene."

For all this set of reasons I suggest here that SRF could emerge as one of the most prominent proteins in the process of KD degeneration, and its sole possible presence inside the nuclear aggregates would confirm this hypothesis. The reason for this is that its recruitment inside the AR fibrils of KD would prevent SRF from performing its proper function and so it would not be able to prevent both skeletal muscle and motor neuronal death and degeneration under toxic events such as the ones that the mutant AR itself could be inducting.

From this point, I also sought to understand exactly what would be the effects of SRF impairment in the motor neurons and muscle cells affected in KD. These could be mainly related to the overexpression of caspase 3, as SRF would be unable to regulate its transcription properly. Moreover, this protein has already proved to be critical in KD as the mutation of the AR causes it to be susceptible for cleavage by this protease ([18]). However, if increased levels of this protein were found they would directly suggest other sources of critical toxic effects for the diseased cells.

Finally, by analyzing the consequences that the increase in caspase-3 could be having it was wanted to be confirmed whether the full-sized or the cleaved form of the AR was necessary to form the aggregates along with other hallmarks related to the evolution of these fibrils with the pass of time.

The techniques used to analyze these set of hypothesis were **Western Blotting** (see "Materials and Methods") along with **computational analysing**.

For one of the studies, 3 different samples from *Mus Musculus* muscle tissue were used containing 97Q, 24Q and wild type (WT) AR respectively (**Note:** #Q accounts for the

[18] Ellerby, L M, A S Hackam, S S Propp, H M Ellerby, S Rabizadeh, N R Cashman, M A Trifiro, et al. "Kennedy's Disease: Caspase Cleavage of the Androgen Receptor Is a Crucial Event in Cytotoxicity."

number of Glutamines from the polyQ tail of AR) to analyze the levels of SRF and caspase-3 there. The mouse containing the 97Q form of the protein served as a model for the mutant form of the AR present in the disease.

Apart from this, the fibrils from the different mice were isolated from the rest of the cellular content to analyze whether or not SRF was present there. In case of the 24Q and WT the structures extracted were not the aggregates present in the disease but non toxic oligomers usually found in normal cells. These were used to seek the amounts of SRF present in each of the cases so as to test whether the recruitment of the protein to the 97Q AR fibrils was actually a real tough aberrant complex or the result of a non selective interaction due to the presence of the protein all around the nuclear content.

In the other of the studies, four mice samples from both the spinal cord and muscle of 7 week and 18 week old mice were used to compare the growth in the amount of SRF recruited in the AR fibrils with time and also to observe the changes these structures underwent. For that reason, the fibrils in all the cases were also needed to be isolated and analyzed.

4. MATERIALS AND METHODS

4.1-TISSUE HOMOGENATES' PREPARATION

Description of the technique

This method was used so as to allow the proper preparation of the tissues to be studied. Here, the cell membranes from mice muscle tissues were disrupted and the intracellular content was homogenized with the aid of a special buffer. Finally, the proteins were separated from the rest of the solution with a centrifuge, as these are the molecules of interest for this study.

Materials

-Mice tissues from:

SPINAL CORD	MUSCLE
7 week old mice containing the 97Q AR	7 week old mice containing the 97Q AR
18 week old mice containing the 97Q AR	18 week old mice containing the 97Q AR
	18 week old mice containing the 97Q AR
	18 week old mice containing the 24Q AR
	18 week old mice containing the WT AR

-7 Eppendorf tubes used so as to insert the different tissue contents

-2 ml of homogenizing buffer per tube:

CONTENT (in 2 ml)

Aprotinin (prevents protein degradation)	12 µl
Acid protease inhibitor (inhibits proteins' breakage by acid protease)	20µl
Cellytic-M mammalian cell lyses reagent (causes the cells' breakage)	2ml
	2 ml

-A 1ml tissue grinder

-A centrifuge

-The Bio-Rad DC total protein content assay kit

-A 1-20 µl graduated pipette

-A 5 ml pipette

-A mixer

Protocol

1. 10% dissolved tissue homogenates were prepared inside the different Eppendorf tubes. 0.1 g of muscle tissue were used in each of the cases in combination with homogenizing buffer.
2. Equations used to set the amounts required in the homogenizing buffer

The acid protease inhibitor was set to a 1:100 concentration with the cell lyses reagent.

$$\frac{1\ \mu l\ acid\ protease\ inhibitor}{100\ \mu l\ cell\ lyses\ reagent} = \frac{x\ \mu l\ acid\ protease\ inhibitor}{2000\ \mu l\ \ cell\ lyses\ reagent}$$

$$x\ \mu l\ acid\ prot.inhibitor = \frac{2000\ \mu l\ cell\ lys.reagent * 1 \mu l\ acid\ prot.inhibitor}{100\ \mu l\ cell\ lys.reagent}$$

$$x\ = 20\ \mu l\ acid\ protease\ inhibitor$$

For aprotinin, a 3:500 concentration was used with respect to the previous mixture.

$$\frac{3\ \mu l\ aprotinin}{500\ \mu l\ (PMSF + cell\ lyses\ reagent)} = \frac{x\ \mu l\ aprotinin}{2000\ \mu l\ (PMSF + cell\ lyses\ reagent)}$$

$$x\ \mu l\ aprotinin = \frac{2000\ \mu l\ (PMSF + cell\ lys.reagent) * 3\ \mu l\ aprotinin}{500\ \mu l\ (PMSF + cell\ lys.reagent)}$$

$$x = 12\ \mu l\ aprotinin$$

3. The exact amounts of aprotinin, acid protease inhibitor and cell lyses reagent were brought together inside each of the Eppendorf tubes.
4. The tissue homogenate was obtained finally with the aid of a 1 ml tissue grinder

(see Fig. 8.1). A mixture of the total cellular content was formed, which was then incubated on ice for 15 min.

5. The samples were then centrifuged at 2500 rpm for 15 min at 4ºC. Two phases were obtained, the pellet and the supernatant. The latter, where all the proteins were present, was extracted. (See Fig. 5)
6. The Bio-Rad DC total protein assay was then performed to analyze the concentration of protein in the supernatant solutions so as to check whether or not another centrifugation was needed to separate the proteins from other debris.
7. The samples were preserved at -80ºC until further use.

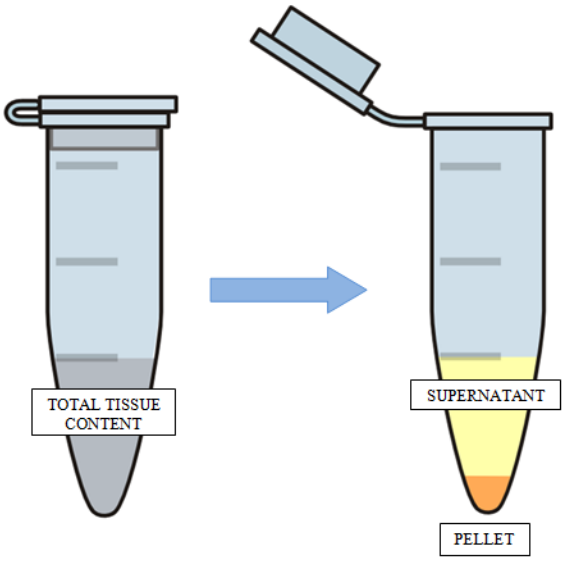


Fig. 5: Schematic representation of the centrifugation procedure. Once the supernatant was obtained, it was isolated from the rest of the debris contained in the pellet (Own elaboration).

4.2-FIBRILS' EXTRACTION

Description of the technique

Here, half of the content from the 18 week old 97Q, 24Q and WT AR-containing muscle samples was taken along with the full content from the other set of samples consisting of either spinal cord or muscle tissue from 7 week and 18 week old 97Q AR-containing mice. Then, the fibrils and the small oligomers present there were isolated from the rest of the protein solution. This was achieved with the aid of a seprion. This special

machine consists of a magnet to which an ionized bead is bound to and that is able to detect amino acid repetitions, as the ones present in the polyQ region of the AR fibrils accounting for its proper separation. This purification was performed several times so as to make sure that no other debris were improperly bound to the seprion.

Materials

-A seprion

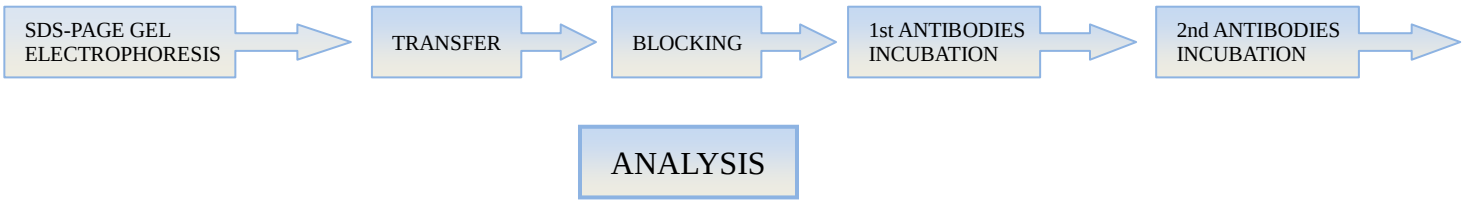
-7 Eppendorf tubes containing the different tissue homogenates.

Protocol

1. From step 4 of the tissue homogenates' preparation, the supernatant of the different Eppendorf tubes was then subjected to the seprion assay that allowed the extraction of the AR fibrils from the rest of the protein content. Although in both the 24Q and wild type cases there were no real aggregates, some AR small aggregated structures could be obtained.
2. The fibrils were then returned to be preserved at -80ºC until further use.

4.3-WESTERN BLOT PROTOCOL

4.3.1-<u>OVERALL STEPS</u>



General overview of the technique

This method was used so as to analyze the presence and also the levels of SRF and caspase-3 inside the different tissue homogenates, along with the changes the AR fibrils

underwent with time. In this technique different wells in a chamber are filled with the different samples and then a voltage is applied which separates the proteins in the wells in a mass-to-charge ratio. This process is known as the gel electrophoresis. Then, these are transferred to a nitrocellulose membrane where specific molecules known as the first antibodies will try to detect the proteins of interest by selectively binding to them. After that, the secondary antibodies will interact with the first ones, and after a special treatment these will emit light that can be detected by the user and that will allow for the sight of the molecules of interest, in case they are present.

As this method consists of multiple parts, its description has been done by separating it into each of the steps it consists on. These are: The gel electrophoresis, the transfer, the blocking, the first antibodies and secondary antibodies incubation and the analyzing.

In this project, four different WB were run.

1. In the first one, the total cellular content of both caspase-3 and SRF was sought to be analyzed in the tissue homogenates from 18 week old mice containing the 97Q, 24Q or WT AR.
2. In the second one, just the presence of SRF was sought to be discovered in the AR fibrils extracted from the aforementioned samples containing the 97Q, 24Q or WT AR.
3. In the third one, a comparative study of SRF presence inside the spinal cord and muscle tissues from both 7 week and 18 week old mice was performed.
4. Finally, a fourth study was developed using the same samples described in the third step but, in this case, the presence of the AR was analyzed with antibodies that detected different domains of this protein (For further information see “The cleaved form of the AR is needed to form the aggregates”)

4.3.2-SDS-PAGE GEL ELECTROPHORESIS

Description of the technique

This technique is used to separate the proteins basing upon their mass-to-charge ratio. The samples were loaded inside the different wells of a chamber containing a polyacrylamide gel while they were also surrounded by a special running buffer. Once a voltage difference was applied between the top and the bottom of the chamber, the proteins started migrating with the aid of the buffer. The gel consisted of different polymers cross-linked with each other that made difficult, in a size-dependent manner, the pass of the proteins through it. By this way, larger proteins were left at the top of the gel while the smaller ones ended up situated at the bottom.

Materials

-450 ml + 5 µl of miliQ water

-50 ml of SDS Tris/Glycine buffer

-3 µl of 6x buffer

CONTENT (in 10 ml)	
1M Tris	3 ml
SDS	1.2 g
β-met	2.4 ml
Glycerol	4.5 ml
H_2O	0.1 ml
Bromophenol	
	10 ml

-The electrophoresis gel

-The SDS-PAGE electrophoresis chamber

-A 1L test

-A 100 ml test

-A 1L flask

-A 1-10 µl graduated pipette

-A magnet

-Eppendorf tubes containing the different samples to be used (They were preserved at -80ºC until further use)

-A mixer

-A heater

Protocol

RUNNING BUFFER PREPARATION: It allows a proper gel run

1. Equation used to set the amounts required

$$450\ ml\ miliQ\ water + 50\ ml\ Tris\ Gly\ buffer = 500\ ml\ solution$$

2. 450 ml of miliQ water were put inside a 1L test so that the exact content could be measured.
3. The same procedure was used with the 50 ml of SDS Tris/Glycine buffer but with a more accurate 100 ml test.
4. The content was brought together into a measured 1L flask thus reaching the 500 ml limit.
5. A magnet was used so as to mix the content.
6. The mixture was put inside the refrigerator at 4ºC until further use.

SAMPLE PREPARATION

1. Equations used to set the amounts required (Total content = 18 µl)

$$18\ \mu l\ total\ solution = 6x * x\ \mu l\ buffer$$

$$x = \frac{18\ \mu l\ total\ solution}{6x} = 3\ \mu l\ of\ buffer$$

$$18\ \mu l - 3\ \mu l = 15\ \mu l\ of\ overall\ sample$$

$$15\ \mu l\ of\ overall\ sample = 10\ \mu l\ of\ sample + 5\ \mu l\ of\ miliQ\ water$$

2. 10 µl of the content from the Eppendorf tubes (where the samples were preserved) was brought together with 3 µl of special 6x buffer and 5 µl of miliQ water with the help of the pipette. This buffer acts as a denaturalizing and ionizing agent. This means that it aids in the loss of the native conformation of proteins and charges them negatively.
3. The content of the Eppendorf tubes was mixed with a mixer.
4. The Eppendorfs were heated up to 95 ºC for 5 minutes in a heater so that the 6x buffer added was able to denaturalize the proteins and ionize them with negative charges. This would allow a proper gel run.

GEL RUNNING

1. The gel was placed inside the electrophoresis chamber and the surroundings and left spaces were filled with the running buffer.
2. The samples were loaded inside the wells of the gel.
3. A safety lid was placed over the electrophoresis' chamber making sure that the leads were plugged inside the jacks of the power supply.
4. The SDS-PAGE electrophoresis was run (Conditions: 200V and 25 min).
5. The remaining running buffer was extracted out of the chamber once the process had finished.

4.3.3-TRANSFER

Description of the technique

In this process, the proteins previously separated in the gel were transferred to a

nitrocellulose membrane that allowed the primary antibodies to interact selectively with the molecules of interest as this is something that could not be achieved if they were just left to act upon the gel.

Materials

-800 ml of miliQ water

-100 ml of Tris/Glycine buffer

-100 ml of methanol

-A 1L test

-A 100 ml tests

-A 1L flask

-A magnet

-The resultant running gel from the electrophoresis

-Fiber pads

-Filter papers

-A nitrocellulose membrane

-A transfer chamber

-Ponceau Staining

Protocol

TRANSFER BUFFER PREPARATION:

It accounts for a proper transfer

1. Equation used to set the amounts required

$$800\ ml\ miliQ\ water + 100\ ml\ Tris\ Gly\ buffer + 100\ ml\ methanol = 1L\ sol$$

2. 800 ml of miliQ water were put inside a 1L test so that the exact content could be measured.
3. The same procedure was applied with the 100 ml of Tris/Glycine buffer and methanol but with two different, more accurate 100 ml tests.
4. The mixtures were brought together into a measured 1L flask thus reaching this limit.
5. A magnet was used so as to mix the content of the recipient.
6. The flask was preserved in a refrigerator set at 4ºC until further use.

TRANSFER RUNNING

1. The fiber pads and the filter papers isolate the transfer run from the environment. These were soaked in transfer buffer thus preventing that no bubbles were left in between the spaces of the fibers and that would cause an improper run.
2. The nitrocellulose membrane was put over the gel in the direction of the electric current so that the proteins were able to migrate to its surface once the voltage had been applied. These also had to be surrounded by the filter papers and the fiber pads in the way that follows:

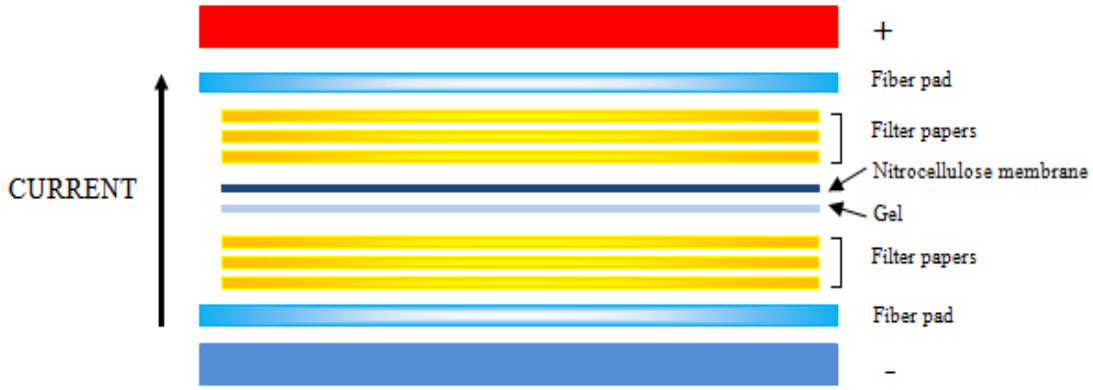


Fig. 6: Schematic representation of the transfer preparation. The gel and the membrane were put together so that the proteins could migrate from one surface to the other being surrounded at the time by the filter papers and the sponges so as to isolate the chamber (Own elaboration).

3. Everything was then inserted inside the transfer chamber.
4. The rest of the spaces inside the chamber were filled with miliQ water and transfer buffer.
5. Ice was also used to avoid heating and for the achievement of better results.
6. A voltage was applied inside the chamber by switching it on to perform the transfer of the proteins from the gel to the nitrocellulose membrane (Conditions: 25 V and 1:15 h)
7. The remaining miliQ water and buffer were extracted once the process had finished.
8. The membrane was stained with Ponceau staining* so that it was possible to see all the protein bands and whether or not the transfer had worked properly.

4.3.4-BLOCKING

Description of the technique

Here, a 5% milk solution (also known as blocking buffer) was applied upon the nitrocellulose membrane. By this way, all the spaces in the membrane that had not been occupied by proteins would be then filled with the buffer thus allowing a selective binding of the first antibodies, which would be applied afterwards, with just the proteins.

Materials

-The nitrocellulose membrane from the transfer

-2.5 g of milk powder

-50 ml of TBS

-A container case

-A falcon

-A mixer

-A reciprocating machine

-A 25 ml graduated pipette

-A balance

Protocol

BLOCKING BUFFER PREPARATION

1. Equation used to set the amount of milk powder required

$$5\%\ milk\ required = \frac{5\ g\ milk}{100\ ml\ TBS\ solution} = \frac{x\ g\ milk}{50\ ml\ TBS\ solution}$$

$$x = \frac{5\ g\ milk * 50\ ml\ TBS\ solution}{100\ ml\ TBS\ solution} = 2.5\ g\ of\ milk\ powder$$

2. Both the exact content required of powder milk (2.5 g) and TBS (50 ml) were brought together with the help of a balance and the graduated pipette inside the falcon.
3. The solution was mixed with the help of a mixer until no insoluble traces of milk were being visible.

BLOCKING RUN

1. A container case was filled with the blocking buffer and the nitrocellulose membrane resulting from the transfer was put inside.
2. This was let remain for 1h in a reciprocating machine until all the spaces of the membrane that had not been occupied by proteins were filled with this buffer.

4.3.5-FIRST ANTIBODIES' INCUBATION

Description of the technique

Here, the different selective antibodies' solutions that detected either caspase-3, AR or SRF were applied upon the nitrocellulose membrane. By this way, these could bind selectively to just the aimed proteins of the study thus allowing their detection in the next steps of the process.

Materials

-10 µl of the first antibodies selective for the different proteins of interest

-5 ml of 5% milk blocking buffer (see "Blocking buffer preparation")

-The nitrocellulose membrane from the blocking

-A container case

-A 1-10 µl graduated pipette

-100 ml of TBS

-100 µl of Tween

-A 100 ml flask

-A mixer

Protocol

1. Equation used to set the amount of 1st antibody required

The first antibody was set at a 1:500 solution with the 5% milk blocking buffer.

$$\frac{1\ ml\ 1st\ antibody}{500\ ml\ milk\ solution} = \frac{x\ ml\ 1st\ antibody}{5\ ml\ milk\ solution}$$

$$x = \frac{5ml\ milk\ solution * 1\ ml\ 1st\ antibody}{500\ ml\ milk\ solution} = 0.01\ ml\ 1st\ antibody$$

$$x = 10\ \mu l\ 1st\ antibody$$

2. The 10 µl of the first antibody were mixed (with the use of the properly graduated pipette) with the 5ml of the 5% milk blocking buffer
3. The nitrocellulose membrane was taken and mixed in a container case with the 5 ml antibody solution. This was then incubated during overnight in a refrigerator set at 4ºC.
4. Equation used to set the amounts of Tween and TBS required

$$\frac{1\ \mu l\ Tween}{1000\ \mu l\ TBS} = \frac{x\ \mu l\ Tween}{100000\ \mu l\ TBS}$$

$$x\ \mu l\ Tween = \frac{1\ \mu l\ Tween * 100000\ \mu l\ TBS}{1000\ \mu l\ TBS}$$

$$x = 100\ \mu l\ Tween$$

5. A 100 ml flask was prepared with a mixture of 100 µL of Tween and 100 ml of TBS (also known as TBST) with the aid of a mixer.
6. The nitrocellulose membrane was washed in a case for 3 times in TBST during 5 minutes each, as this solution is able to remove the antibodies that have non-specifically bound to the proteins of interest, thus reducing the background. This is due to the fact that the antibodies that had not bound to the proteins to which they are selective can interact with Tween and be detached from the membrane.

4.3.6-SECONDARY ANTIBODIES' INCUBATION

Description of the technique

This technique accounted for the proper sight of the protein bands were the first antibodies had bound, as the secondary antibodies bind selectively to the first ones and they contain an enzyme (Horseradish peroxidise, HRP) which catalyzes an oxidation reaction that emits light once the proper reactants are applied (see Analyzing).

Materials

-5 µl of the secondary antibodies selective for the first ones.

-10 ml of 5% milk blocking buffer (see "Blocking buffer preparation")

-The nitrocellulose membrane from the first antibodies' protocol

-A container case

-A 1-10 µl graduated pipette

-100 ml of TBS

CONTENT (in 100 ml)

NaCl	1.1 g
Tris	0.76 g
MiliQ water	100 ml
	100 ml

-100 µl of Tween

-A 100 ml flask

-A mixer

Protocol

1. <u>Equation used to set the amount of 2nd Ab required</u>

The secondary antibody was set at a 1:2000 solution with the 5% milk blocking buffer.

$$\frac{1\ ml\ 2nd\ antibody}{2000\ ml\ milk\ solution} = \frac{x\ ml\ 2nd\ antibody}{10\ ml\ milk\ solution}$$

$$x = \frac{10\ ml\ milk\ solution * 1\ ml\ 2nd\ antibody}{2000\ ml\ milk\ solution}$$

$$x = 0.005\ ml\ 2nd\ antibody = 5\ \mu l\ 2nd\ antibody$$

2. 5 µl of the secondary antibody were mixed (with the use of the properly graduated pipette) with the 10 ml of the 5% milk blocking buffer.

3. The nitrocellulose membrane was incubated with the secondary antibody solution at room temperature for 2:30 h.
4. Equation used to set the amounts of Tween and TBS required

$$\frac{1\ \mu l\ Tween}{1000\ \mu l\ TBS} = \frac{x\ \mu l\ Tween}{100000\ \mu l\ TBS}$$

$$x\ \mu l\ Tween = \frac{1\ \mu l\ Tween * 100000\ \mu l\ TBS}{1000\ \mu l\ TBS}$$

$$x = 100\ \mu l\ Tween$$

5. The mixture of Tween and TBS (TBST) was prepared inside a 100 ml flask with the aid of a mixer.
6. The nitrocellulose membrane was then washed in TBST for 3 times during 5 minutes each thus removing the secondary antibodies that had non-specifically bound to the first ones.

4.3.7-ANALYZING

Description of the technique

Here, two types of reagents were applied upon the membrane; luminol and peroxide. These served as reactants for the enzyme attached to the secondary antibodies (HRP) which then catalyzed an oxidation reaction of luminol emitting light in the process. This light could be then impressed upon a film, as in a photograph, that when treated properly in a revealing machine allowed the sight of the bands.

Fig. 7: HRP oxidation reaction. The reaction leads to the emission of light at 420 nm (Own elaboration).

Materials

-The nitrocellulose membrane from the secondary antibodies' protocol.

-A cassette

-Films

-Aluminium foil

-1 ml of luminol enhancer solution

-1 ml of peroxide

-An Eppendorf tube

-A 0.2-1 ml pipette

-Ethanol

Protocol

1. 1 ml of luminol enhancer solution and peroxide were brought together inside an Eppendorf tube.
2. The Eppendorf content was added with a properly graduated pipette upon the nitrocellulose membrane set over the aluminium foil. 4 minutes were required for the reactants to be converted into the resultant products with the consequent light emission.
3. The excessive reactants were removed from the membrane.
4. The cassette was first washed with ethanol and the membrane was put inside it along with one film (a dark room was used for this purpose so that the film could not be disrupted due to light).
5. After some minutes the film was taken out of the cassette and put into a revealing machine that allowed the sight of the protein bands.

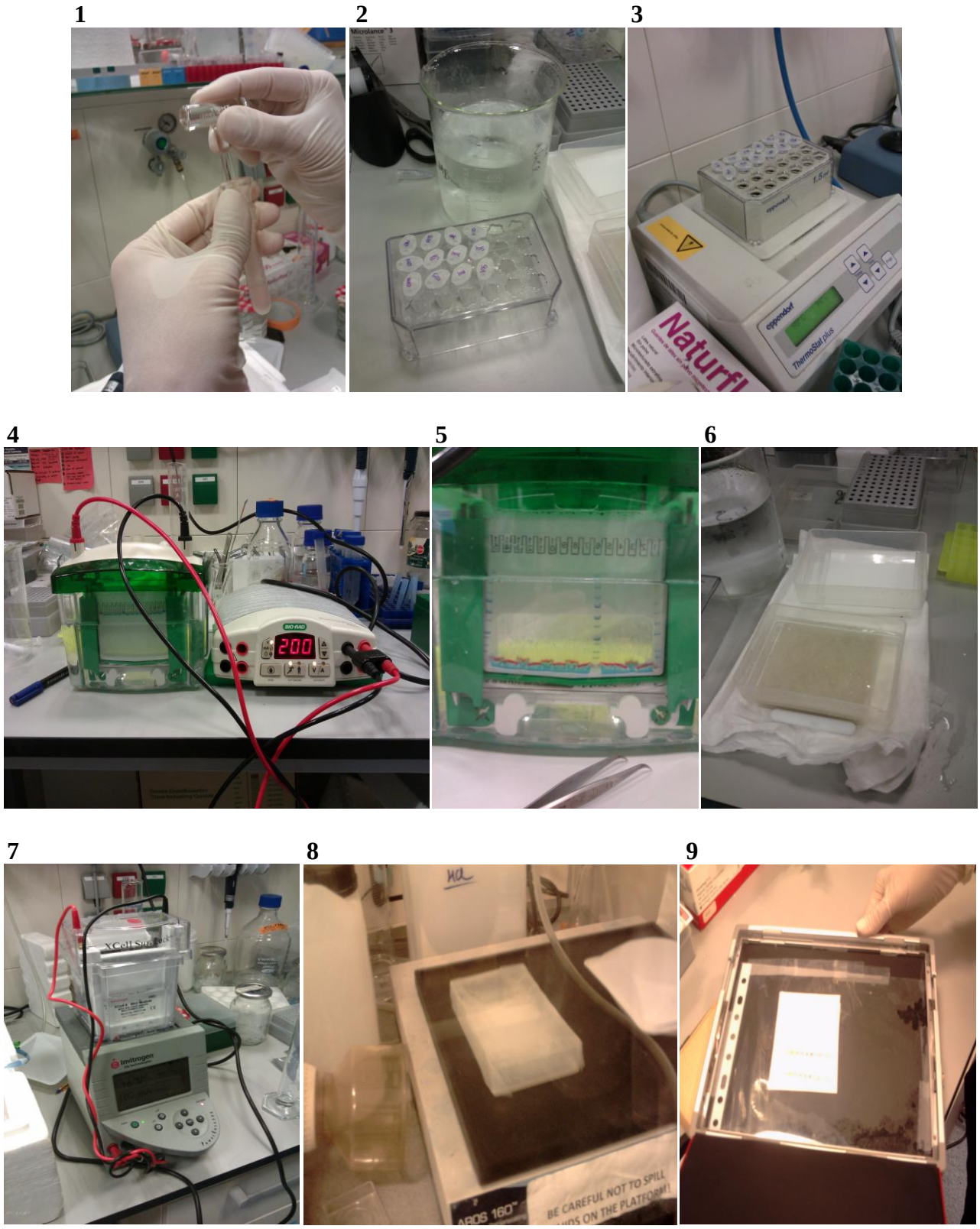


Fig. 8: Graphical resume of the protocol followed. First of all, the tissue homogenates were prepared with a grinder (1). The samples obtained can be observed in image 2. These were heated up so as to be denatured with the aid of a special buffer, as shown in image 3 and the electrophoresis was run (Images 4 and 5). The transfer followed this step. First of all the filter papers and fibers used for this part were soaked in buffer (6) and the transfer was run afterwards (7). In image 8, the process of incubation applied for the blocking, first and secondary antibodies can be observed. Finally, the cassette used for the analyzing is shown in image 9 along with the resultant film from the previous steps (Own elaboration).

5. RESULTS

5.1- SRF IS PRESENT INSIDE THE 97Q AR FIBRILS

The results obtained from the fibril comparison WB, where the levels of SRF in the AR aggregates from 97Q, 24Q and WT AR-containing mice were analyzed, support the hypothesis suggesting a possible presence of SRF inside the 97Q AR aggregates. As shown in Fig. 9, there was found a six-fold increase in this transcription factor levels inside the fibrils extracted from 97Q AR-containing mice with respect to the oligomers taken from the tissues with the 24Q AR. However, the important results come from the fact that high levels of SRF were detected inside the AR oligomers from WT mice too. The ratio between the 97Q AR models and these tissues was of 1.5:1.

There was no remarkable difference between the total cellular amount of SRF in the 97Q, 24Q and WT AR-containing mice. For that, the mean value of the three total tissue contents was taken and compared with the one from the mutant mice fibrils. As it can also be observed in Fig. 9, the levels of SRF inside the aggregates from the 97Q AR corresponded to 0.51 of the total mean cellular content of this protein. This stands for half of the total tissue content being present in the aggregates of diseased mice.

However, the high levels of SRF inside the WT models seem to be contradictory. This effect could be due to the fact that the mice used for the study were old and it had already been reported that AR aggregates could be formed in some individuals even without the need for the mutation ([19]). Despite this, what had not yet been found was that these structures could also be recruiting proteins, as it had been shown in the study. In this case, although, the interaction may be reversible and for that it may not be

[19] Jochum, Tobias, Manuela E. Ritz, Christoph Schuster, Sarah F. Funderburk, Katja Jehle, Katja Schmitz, Falko Brinkmann, Michael Hirtz, David Moss, and Andrew C.B. Cato. "Toxic and Non-toxic Aggregates from the SBMA and Normal Forms of Androgen Receptor Have Distinct Oligomeric Structures."

exerting the toxic effects seen in the mutant mice. The same, however, should be seen in the 24Q AR models as they did not show signs of degeneration either, and it is not. For that, more research needs to be done.

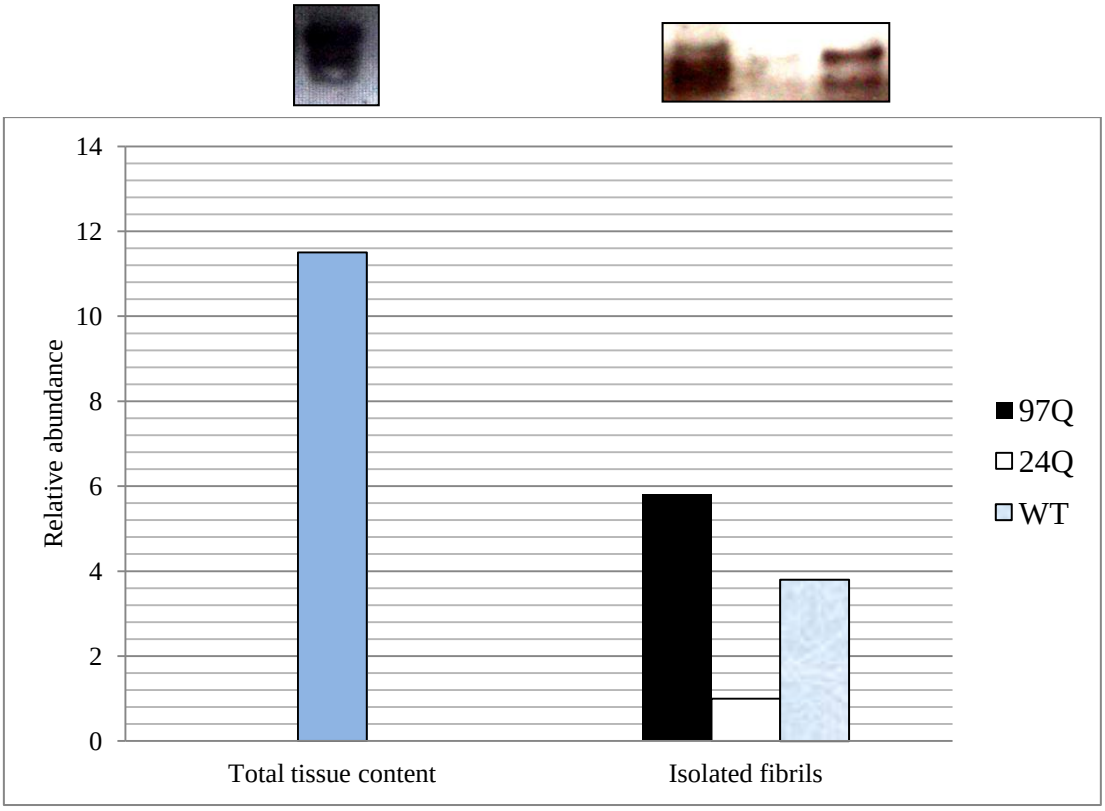


Fig. 9: Serum Response Factor relative amount plot. As it can be observed, a six-fold increase was found in the 97Q AR isolated fibrils with respect to the 24Q although there is not an extensive difference in the WT case (Own elaboration).

5.2-THE LEVELS OF SRF INSIDE THE AGGREGATES GROW WITH AGE

From the results described in the previous section, it was wanted to be solved whether or not with the progressive growth of the fibrils from the diseased mice there was actually an increased recruitment of SRF there. To do this, two sets of fibrils from the spinal cord and the skeletal muscle of both 7 and 18 week old mice were compared.

As seen in the graph from Fig. 10, the results confirm this hypothesis. In the spinal cord, a 4-fold increase was found between the young and old animals. Despite this, in case of the muscle, the growth is not as remarkable as in the other example (1.3:1 ratio) but the

levels of SRF inside the fibrils from this tissue in young animals were 1.29 times higher than the ones in the spinal cord of the eldest.

This suggests that there may be two different rates of fibrils growth and protein recruitment depending on the type of cells. This could be supported by the fact that the involvement of both SRF and AR in muscle cells is especially important during its development stages ([13]), and could point out to the fact that the aggregation in the muscle would be earlier and higher than in other tissues such as the spinal cord (For further evidence about this fact see "The cleaved form of the AR is needed to form the aggregates").

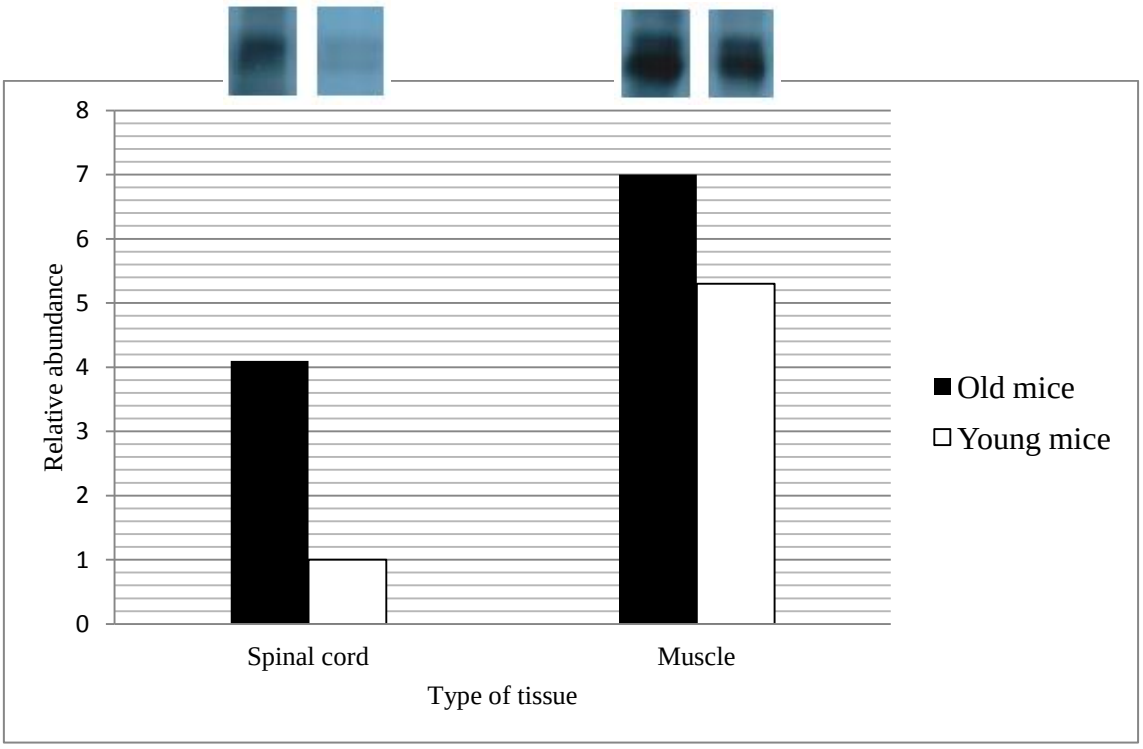


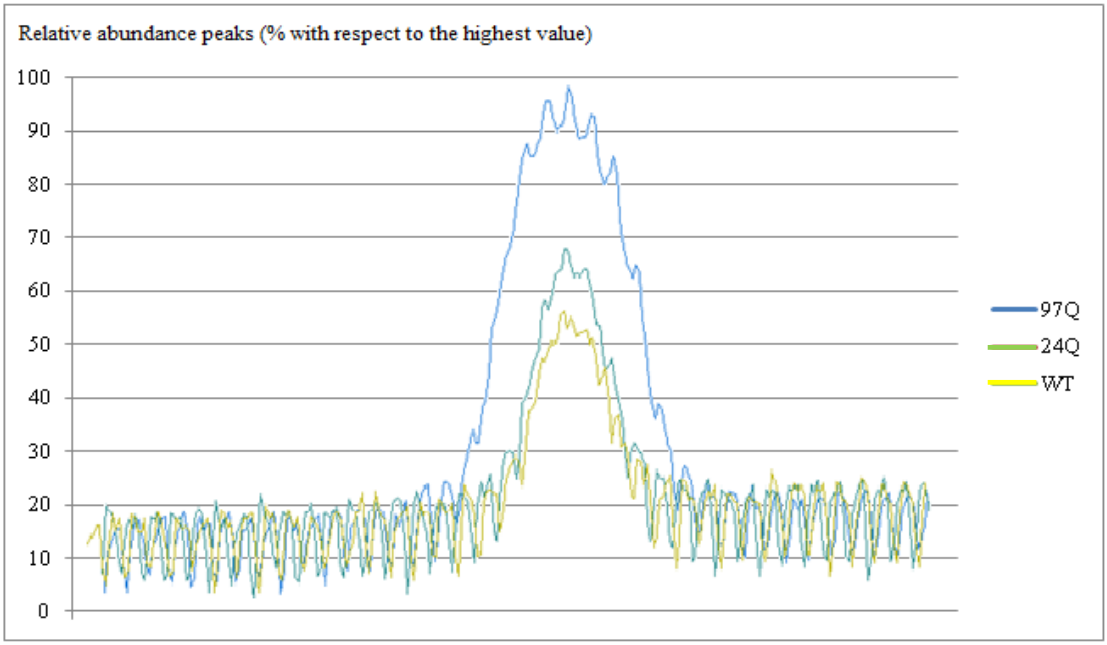
Fig. 10: Graph plotting the relative amounts in the different fibrils. The reference has been taken from the spinal cord young tissue so that all the relative amounts in the graph can be compared. As it can be observed, SRF was practically inexistent in the Spinal Cord (SC) from young mice, and even in case of the tissue extracted from old animals, a higher amount was found in fibrils extracted from young muscle tissue (M) at a ratio of 1.4:1(Own elaboration).

5.3-CASPASE-3 LEVELS ARE ALSO INCREASED IN MUTANT MICE

From this point, I sought to discover what the consequences of SRF presence inside the

aggregates would be. SRF is a major regulator of caspase-3 and this protein is causing the breakage of crucial molecules into fragments, thus causing the loss of their function, in cells damaged due to the action of toxic stimulus (apoptotic conditions). For that, its activity is crucial for cellular survival and degeneration. Moreover, in some studies it had been pointed out that the mutation in AR could cause this protein to be susceptible for cleavage by caspase-3 in the disease ([18]). However, if a shift to higher levels of this protease were found in the total cellular content of diseased mice, they would directly suggest other sources of toxic consequences in KD, apart from just the cleavage to the AR, and related to the disruption of other critical proteins' activity.

As shown in the peaks of Fig. 11 and 12, where the highest of them stands for the 97Q AR tissues, the results obtained from the Western Blot support the fact that, when analyzing the total cellular content, caspase-3 had higher levels in the tissues extracted from diseased mice with respect to both the two other ones at a ratio of 3.2:1.4:1 for the 97Q, 24Q and WT AR models respectively.



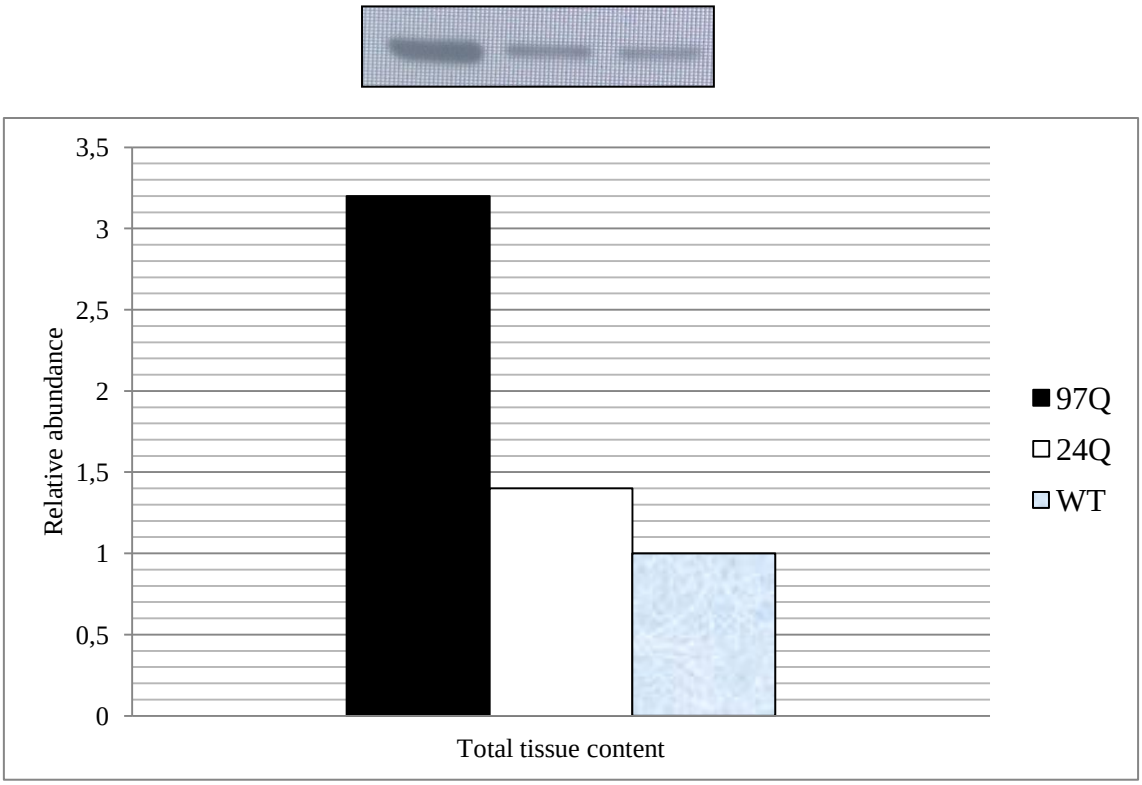


Fig. 11-12: Caspase-3 levels are shifted towards a higher level in the 97Q AR-containing mice. In case of the first representation, the blue peak corresponds to the 97Q AR, the gree one to the 24Q AR and the yellow one to the WT mice. In the second one, the exact amounts were plotted and it can be observed that caspase-3 is 3.2 times higher in the diseased models with respect to the WT (Own elaboration).

These results, with that, suggest that caspase-3 may not be only having an increased activity against the mutant AR, but also it may be acting against other targets of the cell machinery due to its increased levels. This is something that is even more strongly confirmed in the next section. (See Caspase-3 activity is also increased in the disease).

5.4- CASPASE-3 ACTIVITY IS ALSO INCREASED IN THE DISEASE

Caspase-3 is activated by being cleaved into two fragments. The fragments are structured into a dimer that further binds with another one thus forming a fully functional structure. Only after this process has occurred is caspase-3 able to perform its function. For that reason, here I sought to discover whether or not the levels of cleaved protein where higher than in normal conditions in the diseased mice. If that was true, it would directly confirm that not only are the levels of caspase-3 increased in the disease,

but also that the active form of the protein is present there, acting against different targets of the cell. As the cleaved form of caspase-3 has a reduced size, the band corresponding to this peptide in the WB would be present below the one corresponding to the full-length protein. This band was found and compared in the three different mice muscle tissues from 97Q, 24Q and WT AR-containing mice

As it can be observed from Fig. 13 the results confirm the hypothesis suggested. When analyzing the size of the bands in the 97Q AR- containing mice, it was found that 38 times higher were the levels of cleaved caspase-3 with respect to the WT mice. In case of the 24Q AR-containing models the ratio with the 97Q AR models was set at 2.98: 1.

The importance of this point comes from the fact that it would be not only SRF the one involved in the consequences and direct effects of caspase-3 upon the cell but also other pathways that would be causing the activation of the protein itself. This is due to the fact that, as shown, not only the levels of protease were increased in the diseased mice but also the ones of the cleaved, active, protein. It has been regarded in some studies that so as to be activated, mitochondrial dynamics and function need to be impaired. These organelles are then initiating a signalling pathway that activates a protein known as cytochrome C, which is responsible for the cleavage of caspase-3 ([20]). Moreover, the impairment of mitochondria could be directly related to the mutant AR in KD ([21]), but it cannot be discarded that SRF could be playing a major role there ([15]) (See Fig. 14).

[20] Gorman, A M, E Bonfoco, B Zhivotovsky, S Orrenius, and S Ceccatelli. "Cytochrome c Release and Caspase-3 Activation During Colchicine-induced Apoptosis of Cerebellar Granule Cells."

[21] Piccioni, Federica, Paolo Pinton, Silvia Simeoni, Paola Pozzi, Umberto Fascio, Guglielmo Vismara, Luciano Martini, Rosario Rizzuto, and Angelo Poletti. "Androgen Receptor with Elongated Polyglutamine Tract Forms Aggregates That Alter Axonal Trafficking and Mitochondrial Distribution in Motor Neuronal Processes."

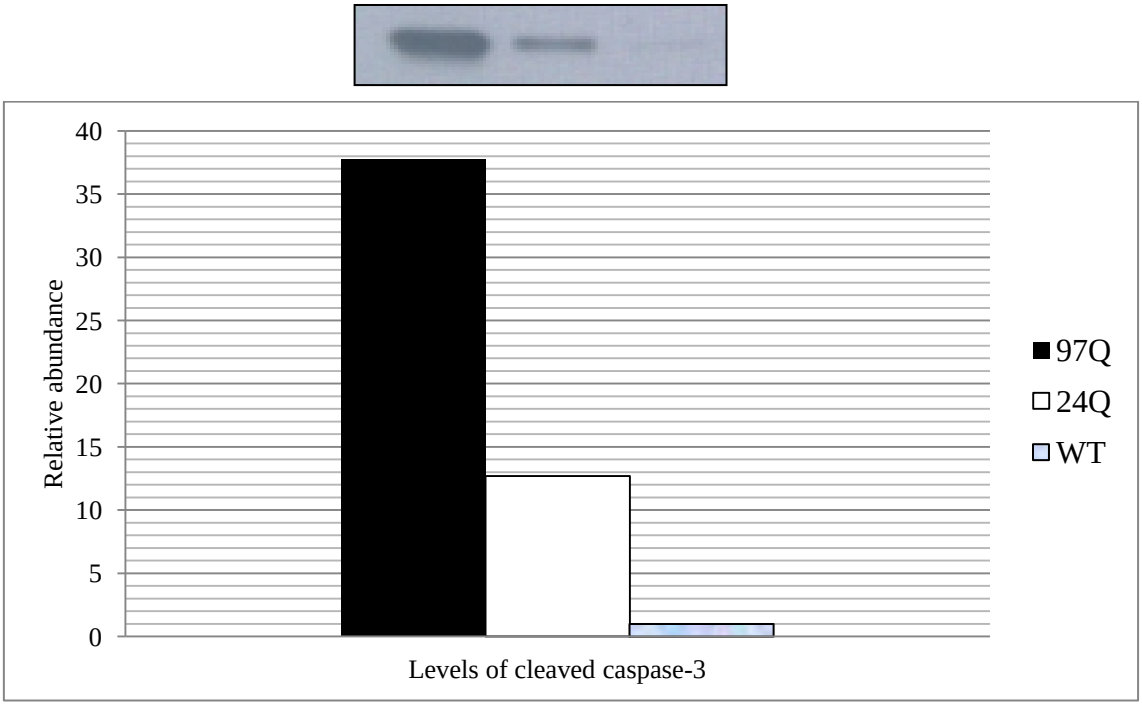


Fig. 13: Graph plotting the relative amounts of cleaved caspase-3 in the different tissues. The ratios plotted are of 38:13:1 (Own elaboration).

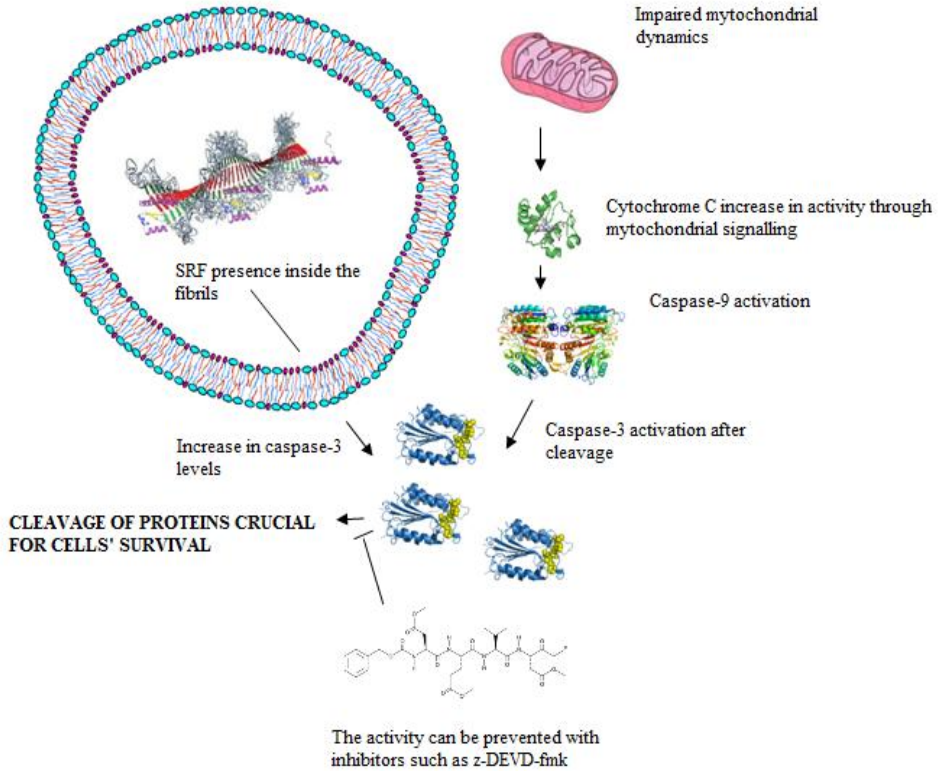


Fig. 14: Caspase-3 activation feedback loop. The cascade could start with the formation of the fibrils where SRF is recruited. This would be causing an increase in caspase-3, further activated by other mechanisms related to mitochondrial impairment (Own elaboration).

5.5-THE CLEAVED FORM OF THE AR IS NEEDED TO FORM THE AGGREGATES

As shown in the previous section, caspase-3 was found to be a feasible candidate to be causing toxic effects for the cell because of the presence of its cleaved, active form, in the diseased cells from the skeletal muscle. Apart from this, there had been also some studies where it had been reported that, in KD, once the AR was mutant and so had a polyQ expanded tail, it was susceptible for caspase-3 cleavage ([18]). For that, here I wanted to analyze whether or not the cleaved form of the AR was necessary to form the fibrils.

To do so, a WB was performed applying two different types of antibodies against AR in a set of samples from both 7 week and 18 week old mice extracted from the spinal cord and the skeletal muscle of both animals. The antibodies selectively detected either the N-terminal or C-terminal* domains of the AR, two areas that, once cleaved, are separated from each other in the different proteolyses' fragments obtained. For that, the possible outcomes of the study would be that:

1. Both antibodies detected the AR fibrils in the WB. This would mean that the full sized protein is present in the aggregates as both the N and C-terminal of the protein would be detecting them.
2. Only the N-terminal antibodies detected the fibrils. This would suppose that a cleaved form of the AR would be forming the aggregates and so the C-terminal antibody would be unable to detect them.

The results obtained confirmed the second hypothesis as the C-terminal antibody was unable to detect any trace of fibrils in neither the spinal cord nor the skeletal muscle

tissues. Despite this, oligomers consisting of AR dimers could be found in the spinal cord tissue (see Fig. 15).

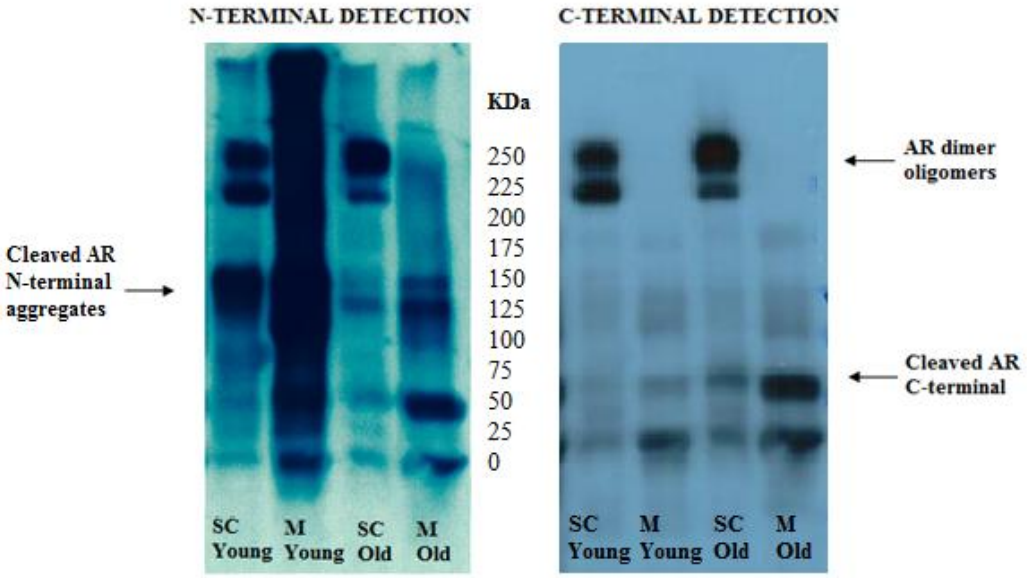
However, another possibility could not be discarded and this is that the C-terminal part of the AR could be hidden inside the protein in an area where it would be unable for the antibody to detect it.

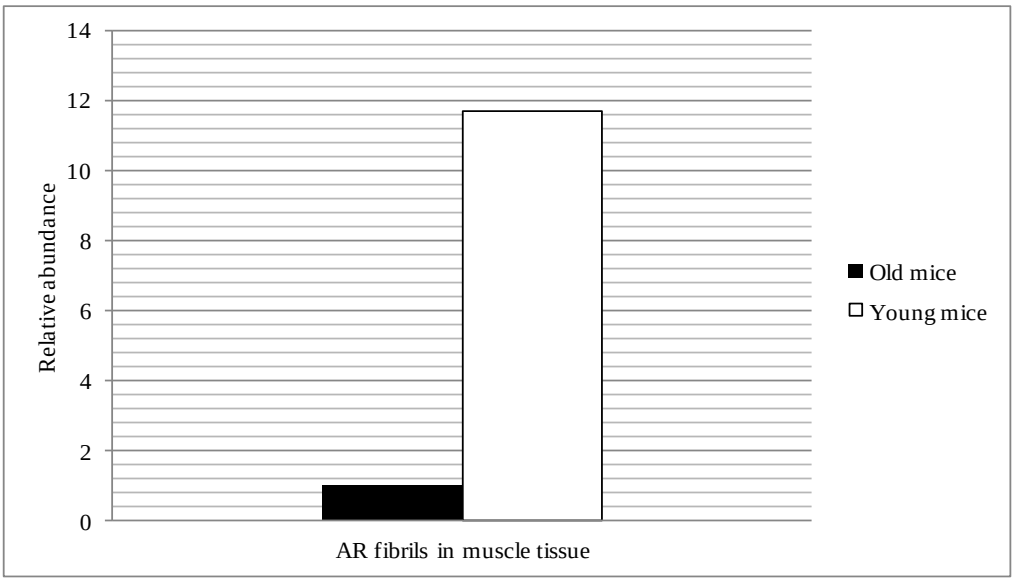
Apart from this, only in the skeletal muscle from young mice could fibrils be found with the N-terminal antibody (see Fig. 15 and 16), but not in the one from the eldest. These results seem to be contradicting what had already been found before, and this was that fibrils were growing with age as more AR was accumulating inside them. In contrast, the levels of the cleaved AR C-terminal were found to be higher in the muscle from old mice (See Fig. 17) which suggests that there may be other mechanisms necessary so as to lead to fibril formation and not just an increased presence of the cleaved protein in the total cellular content.

Finally, in the spinal cord of mice, only oligomers could be found with both antibodies that progressively grew with age (See Fig. 18), but not the aggregates seen in the muscle tissue. This, with that, suggests that these initial aggregated structures can be formed even without the need for the cleavage to the AR. Apart from this, these results are also in accordance with the lower levels of SRF found in the structures extracted from the old mouse spinal cord tissue with respect to the muscle fibrils found in the young one (See “The levels of SRF inside the fibrils grow with age”).

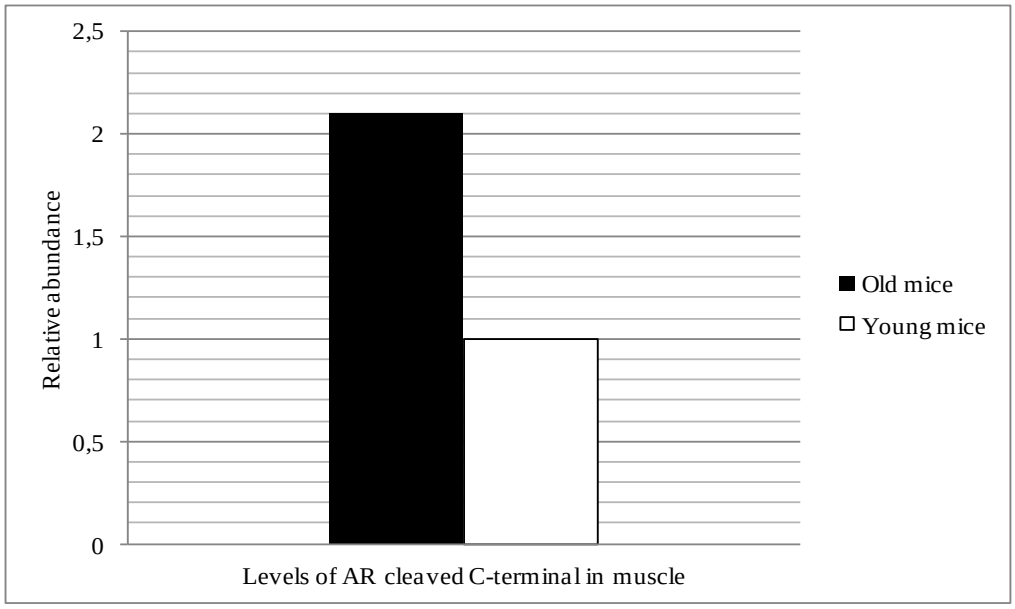
For that, a reason why no fibrils were being present in the former was sought. To do so, the total cellular content of AR in the spinal cord and skeletal muscle from *Mus Musculus* was compared with BioGPS, a database that plots the total mean expression

levels of a wide array of proteins inside the different tissues from these animals ([22]). As shown in Fig. 19, the levels of AR expression in the skeletal muscle were 11 times higher than in the spinal cord suggesting the possible reason why only fibrils were found in the muscle, due to the increased levels of mutant protein there that, with that, would be allowing the formation of the large aggregates seen in mutant mice.





[22] Wu, Chunlei, Camilo Orozco, Jason Boyer, Marc Leglise, James Goodale, Serge Batalov, Christopher L. Hodge, et al. "BioGPS: An Extensible and Customizable Portal for Querying and Organizing Gene Annotation Resources."



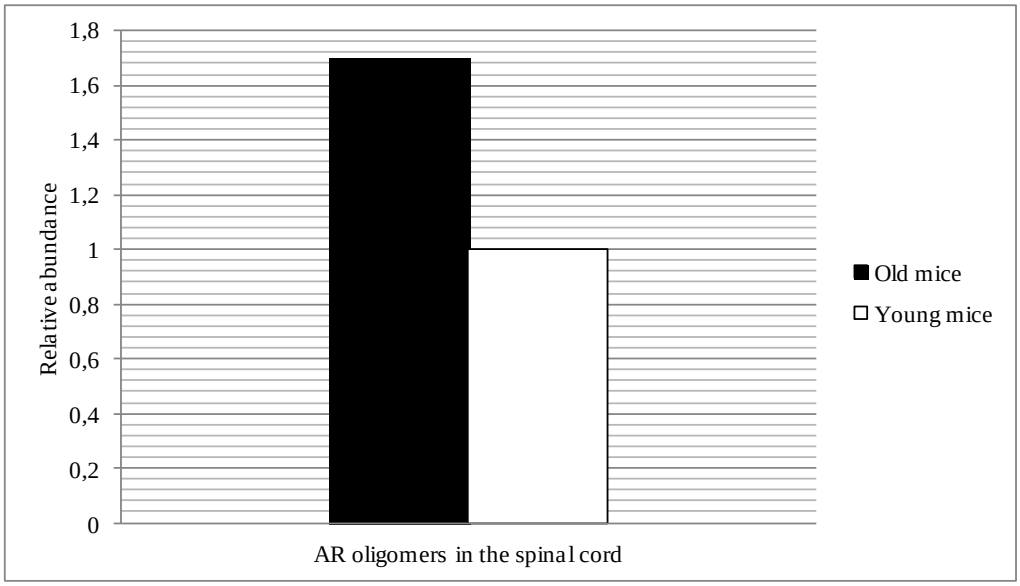


Fig. 15: Two different WB ran to analyze fibril presence in both young and old mice. M stands for muscle and SC for spinal cord. As it can be observed, only fibrils were detected with the N-terminal antibody which suggests that the cleaved form of the AR may be needed to form the fibrils (Own elaboration.

Fig. 16: Graph showing the difference in fibril size from mice muscle tissue. As it can be observed the size of the aggregates was higher in young mice at a ratio of 11,7:1 with respect to the eldest (Own elaboration).

Fig. 17: Schematic representation of the cleaved AR C-terminal levels in muscle tissue. Contrary to what can be seen in Fig. 15 and Fig. 16, the levels of cleaved AR C-terminal were lower in the young mice which suggests that not only cleavage is needed for the fragments to form the aggregates (Own elaboration).

Fig. 18: AR oligomers in the spinal cord. No fibrils were found in the spinal cord but, as seen in the results, the oligomers consisitng of the full-sized AR were 1,7 times higher in the old mice models. This supports the fact that fibrils grow with age, in contrast to the results found for the muscle (Own elaboration).

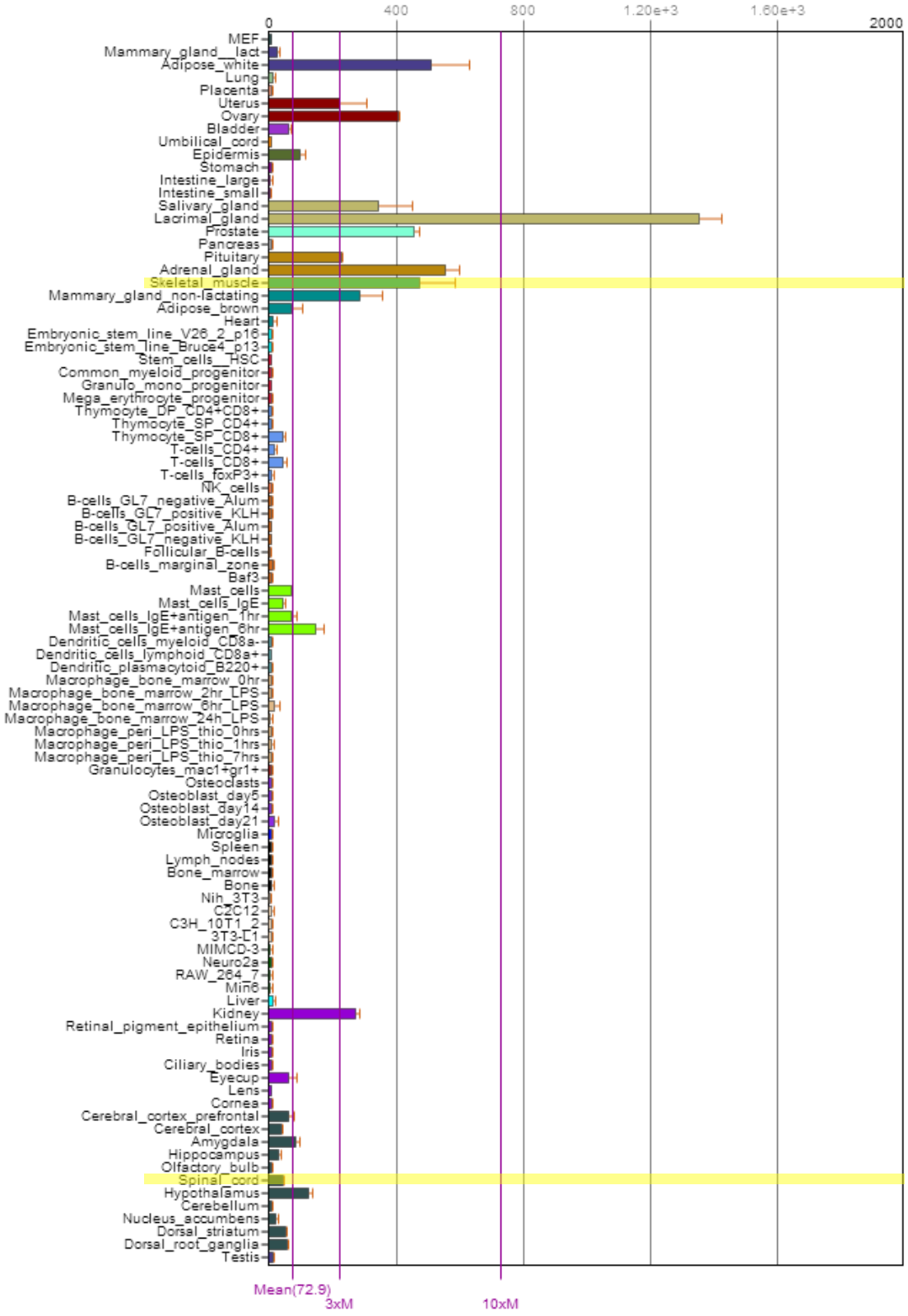


Fig. 19: Gene expression pattern of AR for "Mus Musculus". As it can be observed in the labelled areas, the levels of this protein are much higher in the skeletal muscle than in the spinal cord at a ratio of 11:1 (Own elaboration).

All in all, these results seem to indicate that the formation of AR fibrils may be started with the cleavage of this mutated protein by caspase-3, due to its mutation that makes it susceptible for aggregation and cleavage (See Fig. 20), which would be then allowing it to form oligomers leading to the formation of fibrils. Most likely, the cleavage of this protein into two fragments may be inducting a conformation in the AR prone to form the aggregates. The fibrils would be then recruiting SRF that would be with that causing a rise in caspase-3 levels in a positive feedback loop.

This would be especially important in the skeletal muscle, where the importance of this protein is critical for the proper development of this tissue.

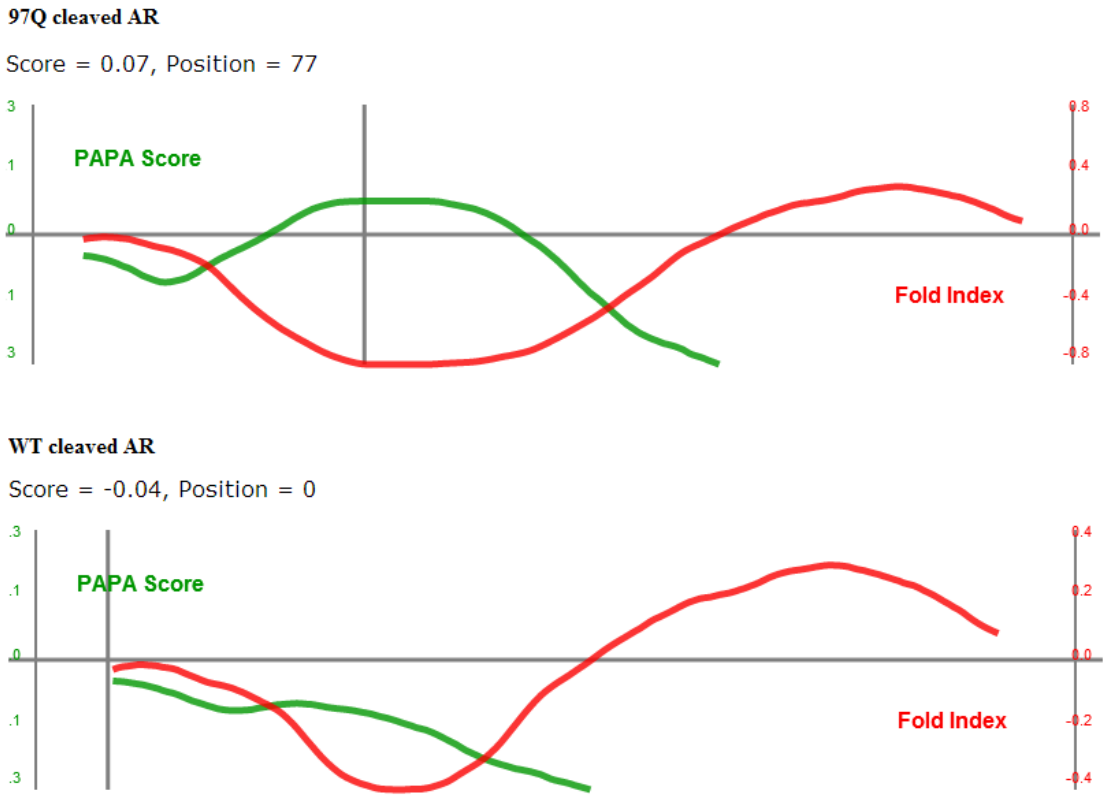


Fig. 20: Comparison of the cleaved AR aggregation likelihood between the mutant and wild-type protein. This comparison was performed with the PAPA software ([23]) that plots the prone a protein is to aggregate basing upon its amino acid sequence. A higher score than 0.05 stands for a high tendency to aggregate and, as it can be seen, the 97Q AR scored 0.07 in the polyQ region. It was also there were the protein was more unfolded (Own elaboration).

[23] Toombs, James A., Michelina Petri, Kacy R. Paul, Grace Y. Kan, Asa Ben-Hur, and Eric D. Ross. "De Novo Design of Synthetic Prion Domains."

6. CONCLUSIONS

The results obtained from this study directly support the set of hypothesis suggested. First of all, as seen above, the amount of SRF present inside the fibrils from diseased mice directly suggest that there could be an inhibition of this protein in the areas affected in KD. This was observed both when analyzing the levels of SRF inside fibrils from the mice with the mutant AR, and also when comparing the rise in the recruitment of SRF inside the AR aggregates as these were progressively growing with age. This not only uncovers that this protein could be undergoing a critical loss of function in KD but is also confirming that, from a general point of view, as AR fibrils progressively grow, more proteins are accumulating inside them causing the induction of worsened toxic events for the cells.

After that, the consequences that this protein's presence inside the aggregates could be having were analyzed by plotting the hypothetical rise in the levels of caspase-3, a protein that had already been regarded to have major importance in the disease due to the mutation in AR that caused it to be susceptible for cleavage. The results obtained directly suggested that not only had caspase-3 higher levels but also that this protein was highly activated in the disease.

Finally, when analyzing what the effects of caspase-3 rise could be, an important hallmark was found and this was that the cleaved form of the AR was the one needed to form the aggregates, that fibril formation did not depended on the amounts of cleaved AR and that these structures could be already formed in early stages of muscle development.

These results seem to indicate that the formation of AR fibrils may be started with the cleavage of this mutated protein by caspase-3, due to the mutation in its polyQ tail that makes it susceptible for cleavage, which would be then allowing it to form oligomers

leading, afterwards, to the formation of fibrils. Most probably, the cleavage of this protein into two fragments may be inducting a conformation in the AR likely to form the aggregates. The fibrils would be then recruiting proteins crucial for cells' survival, such as SRF, that would be with that causing a rise in caspase-3 levels in a positive feedback loop. In this process, the AR would be losing its function causing that testosterone, its natural activator (see "The Androgen Receptor and Testosterone"), would be unable to induce its activation, what is known as androgen insensitivity ([5]). It had already been reported that once this was happening the levels of testosterone rise to be higher than normal ([24]), and this increase caused that the ones of the AR also did so ([25]). The former has also already been detected in KD ([26]), and it has been found too that the overexpression of WT AR recapitulates KD symptoms ([27]). For that, all the process could be ending up with more AR available for fibril formation (see Fig. 21 for a schematic representation).

Apart from this, the loss of SRF function in KD would be also causing other side effects apart from just the caspase-3 levels increase. These would be mainly due to this transcription factor being unable to rescue cell survival under apoptotic conditions and thus leading to apoptosis. Inside this array are included a reduced ability to control α-actin dynamics, which would be consequently causing mitochondrial impairment, a

[24] Brinkmann, Albert O. "Molecular Basis of Androgen Insensitivity."

[25] Krongrad, Arnon, Carol M. Wilson, Jean D. Wilson, Diane R. Allman, and Michael J. McPhaul. "Androgen Increases Androgen Receptor Protein While Decreasing Receptor mRNA in LNCaP Cells."

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[27] Monks, Douglas Ashley, Jamie A Johansen, Kaiguo Mo, Pengcheng Rao, Bryn Eagleson, Zhigang Yu, Andrew P Lieberman, S Marc Breedlove, and Cynthia L Jordan. "Overexpression of Wild-type Androgen Receptor in Muscle Recapitulates Polyglutamine Disease."

reduced microglia activation after injury, an inability to repair axonal damage under toxic events ([28]), and an impairment in skeletal muscle development ([13]). What is more, most of these consequences have been detected in KD ([21]) suggesting the critical role this protein may be playing in the disease.

However, these conclusions are apparently contradicted by the fact that a reduced AR fibril size was found inside the old mice muscle tissue with respect to the one from the younger models. One theory to explain this could be that the involvement of this protein is especially important in the early stages of muscle development ([29]) and so its increased levels and translocation to the nucleus there could be causing the formation of large aggregates. For that, fibrils could be formed then and be progressively degraded with time through the proteasome degradation pathway*, although not completely. This could with that also explain why the levels of SRF did not grow significantly in the muscle tissue from old mice (See "The levels of SRF inside the fibrils grow with age") although if this was completely true, the amounts should be even reduced. It is for that reason that more research needs still to be done around this topic.

Overall, these results are supporting the crucial involvement one single protein could be having in KD along with the importance that some of its consequences could be having for the neurodegenerative course of this disorder. Moreover, with them, a more complete understanding of the AR fibril development process, the most prominent hallmark in the disorder, has been also achieved.

For that, to conclude, I suggest that therapeutic approaches should be directed against

[28] Chang, Sandra H., Steve Poser, and Zhengui Xia. "A Novel Role For Serum Response Factor in Neuronal Survival."

[29] Sinha-Hikim, Indrani, Wayne E. Taylor, Nestor F. Gonzalez-Cadavid, Wei Zheng, and Shalender Bhasin. "Androgen Receptor in Human Skeletal Muscle and Cultured Muscle Satellite Cells: Up-Regulation by Androgen Treatment."

the positive feedback loop described above. Caspase-3 inhibitors such as z-DEVD-fmk ([30]) could be used so as to prevent not only the cleavage by this protease of proteins crucial for cell survival in the disease but also the one of the AR, as this would reduce further fibril growth along with restraining both SRF loss of function and the one of the rest of proteins recruited inside the aggregates. However, there is still also a wide extent of possibilities left targeting the degenerative cycle of this disease and that could be addressed in future research.

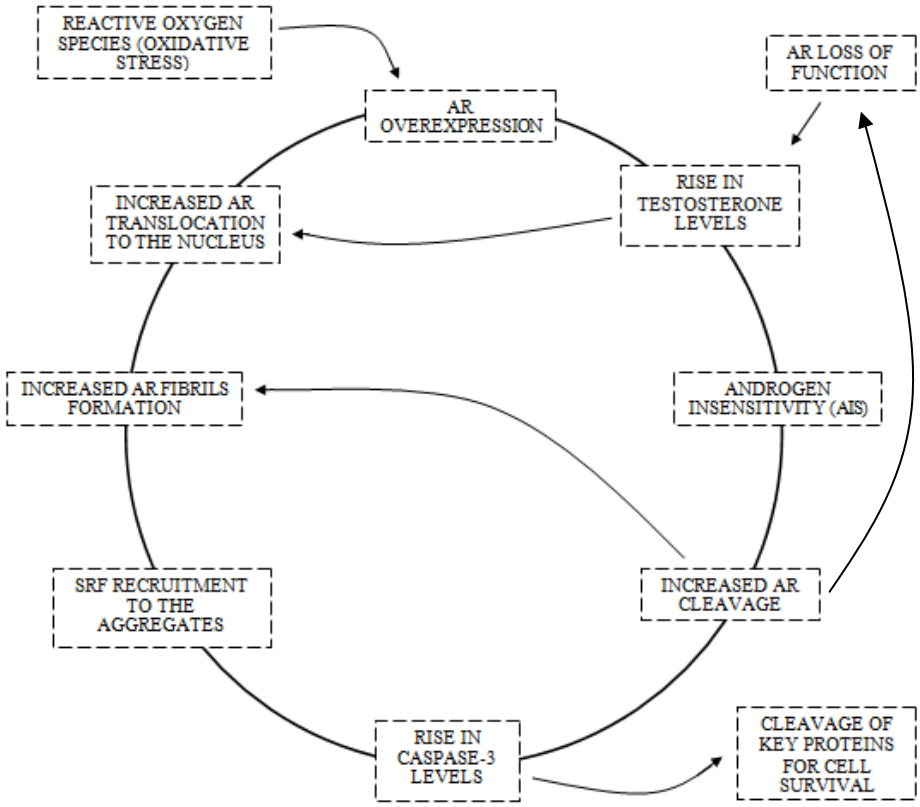

Fig. 21: Frame of Kennedy's disease degenerative process understanding. This hypothetical representation has been drawn from the results obtained in this article that, along with previous reports have led to a more complete understanding of KD degeneration course. The ultimate purpose of this is the future search for therapeutic approaches targeting anyone of the steps described above (Own elaboration).

[30] Knoblach, Susan M, Daniel A Alroy, Maria Nikolaeva, Ibolja Cernak, Bogdan A Stoica, and Alan I Faden. "Caspase Inhibitor z-DEVD-fmk Attenuates Calpain and Necrotic Cell Death in Vitro and after Traumatic Brain Injury."

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8. GLOSSARY

5-α-reductase: Enzyme that catalyzes the reduction reaction of androgens. This means that in the process a double bond is broken through the addition of a hydrogen ion.

Androgen insensitivity syndrome (AIS): Syndrome characterized by an inability of the cells to respond to androgens. It affects both men and women and results in an underdevelopment of their differential treats.

Apoptosis: Process of programmed cell death.

Beta-sheet: Regular domain present in proteins that is formed through the binding of multiple chains in the protein by hydrogen bonds forming a structure resembling a pleated sheet.

Brainstem: Posterior part of the brain involved in the control over the cardiac and respiratory functions. Most of the connections related to stimulus and motor responses pass through this area.

Cleavage: Breakage into fragments of proteins by special molecules known as proteases that leads to a loss of their function. It is critical under apoptotic conditions.

C-terminal domain: Ending domain of a protein that finishes with the free carboxyl (-COOH) group of the last amino acid in the chain.

Dihydrotestosterone (DHT): Resultant product of testosterone reduction by an enzyme

known as **5-α reductase**.

Dimer: Entity constituted by two structurally similar monomers joined by bonds between them.

Glutamine: Non essential polar amino acid.

Kinesins and dyneins: Transport proteins present in neurons that migrate through microtubules and that are responsible for the carriage of essential components for the cell such as mitochondria along their axons.

Ligand-binding domain of AR: Domain of the AR mainly responsible for the interaction with testosterone.

MAPK pathway: Molecular pathway of critical importance for the cells. It involves a family of proteins known as Mitogen-activated protein kinases (MAPK) along with a wide set of others such as Ras, ERK or JNK, depending on the case, that by crosstalking through phosphorylation are responsible for signals including cell survival, transcription or mitosis.

Motor neurons: Neurons that project their axons to the muscle so as to control their response after a stimulus.

N-terminal domain: Starting domain of a protein that initiates with the free amine ($-NH_2$) group of the first amino acid in the chain.

Phosphorylation: Transmission of a phosphate group from one protein to another that inducts a conformational change in the latter leading to either its activation or inhibition.

PolyQ region: Protein domain containing an increased ratio of Glutamine. It has been detected in proteins such as AR, Huntingtin or ataxin-3 and often correlates with a high tendency to form aggregates.

Ponceau staining: Dye used for the detection of proteins present upon a nitrocellulose

membrane.

Protease: Class of enzymes involved in the proteolysis of proteins (That is, their breakage into fragments) through the hydrolysis of their peptide bonds.

Proteasome degradation pathway: Pathway involved in the degradation of damaged proteins into their amino acids through their proteolysis.

RhoA pathway: Molecular pathway dependent on a protein known as Ras homolog gene family, member A and that is widely involved in neuronal repair and survival.

Spinal cord: Long tubular bundle of neurons that serves as a link between the central and peripheral nervous systems. It is mainly related to the transmission of the signals from the brain to the rest of the body.

TCF family of cofactors: Transcription factors that bind to the DNA and that can induct gene transcription.

Testosterone: Molecule mainly present in men and involved in the development of their differential treats. It is mainly exerting its effects through the binding to the AR.

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