Università degli Studi di Padova
Dipartimento di Biologia
Corso di Laurea in Biotecnologie

Elaborato di Laurea

SERCA PUMP AND RELATED PATHOLOGIES: COMPARATIVE STUDY OF DIFFERENT ANIMAL MODELS

Tutor: Prof.ssa Roberta Sacchetto
Dipartimento di Biomedicina Comparata ed Alimentazione

Laureanda: Francesca Nistri

Anno Accademico 2021/2022

Index

1. Abstract

In this work we analysed three pathologies linked to a malfunction of SERCA pumps that gives birth to anomalies during muscles' contraction. After giving a general view on how SERCA pumps work, we focused on three different pathologies. The conditions that we studied are a mutant form of zebrafish known as *Accordion,* PMT in Chianina cattle and human Brody's disease. Considering these three models we could observe how all these pathologies are linked by a malfunction of the SERCA pumps. What is interesting for our study, after an overall observation of the pathologies, are the comparisons that could have been done. What we found out is that both *Accordion* mutant and PMT affected Chianina cattle could be very useful animal models in order to study Brody disease; surprisingly the same can not be said about another common animal model: mice. The conclusion of this study is focused on giving a hypothetical therapeutic approach that takes advance of the functional rescue of mutated SERCA1.

2. Description of the SarcoEndoplasmic Reticulum Calcium ATPase (SERCA) pump

2.1. Role of SERCA in the muscle

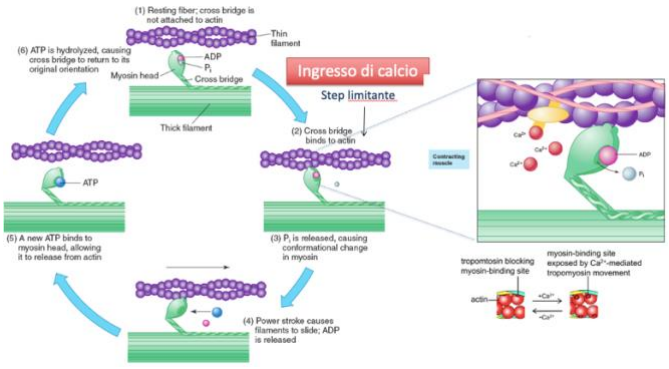
As well known, maintaining homeostasis is fundamental for every organism, particularly calcium homeostasis is critical to maintenance of muscle function. One of the systems that acts as a key regulator of calcium homeostasis is the SarcoEndoplasmic Reticulum Calcium ATPase pump, that from now on we will refer to as SERCA. SERCA pumps belong to a family known as P-type ATPases. The main function of SERCA pump is to permit the uptake of cytosolic Ca^{2+} back into SR lumen taking advantage of the energy derived from the hydrolysis of ATP, at the end of muscle contraction cycle.

Thanks to some studies (Xu and Van Remmen, 2021)[1], nowadays the kinetic cycle of SERCA pump is well known. First, it is important to understand that SERCA exists in two conformational states: E_1, highly affinity to Ca^{2+} ions, and E_2 being low affinity. When Ca^{2+} ions are transported, ATP binding is coupled with the change of these two states. In each cycle, two Ca^{2+} ions and an ATP molecule bind the E_1 state, then the ATP hydrolysis happens and the state is changed to E_2, releasing ADP. While Ca^{2+} ions are released in the SR lumen, an exchange of luminal protons on the E_2 state is required. At the end, when dephosphorylation ad dehydrogenation occurs, the enzyme returns to E_1 state so that a new cycle can start.

2.2. Muscle contraction and calcium's role

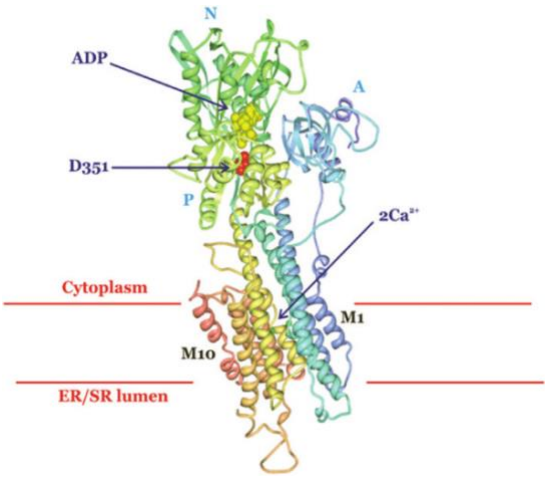
What we just said is that SERCA pumps are fundamental to maintain calcium homeostasis, due to its main role in muscle relaxation.

Now, we are going to do a fast overview on how the skeletal muscle do contract and on how and why calcium is so important in this process.
Skeletal muscles contract on the basis of Huxley's theory, also known as slip theory; this theory manifest itself in three main points: (1) filaments' length is invariant, there is not any kind of elastic protein, (2) sarcomere's length decreases, due to filaments' overlap, and (3) the strength developed is due to the coupled movement of the filaments.
Observing the contraction in detail, we are going to face a process known as crossed-bridges cycle, in which the main actor is calcium itself. The cycle starts from a rest condition in which myosin heads and their binding sites on actin are not associated; in this first condition, crossed-bridges are absent and myosin heads bind ADP + Pi. In order to permit the formation of a crossed-bridge and to trigger the contraction, an increase in calcium concentration is needed.
How does $[Ca^{2+}]$ increase? Following membrane depolarization, the process that permit to raise calcium levels is known as excitation-contraction (EC) coupling. This event is led by two membrane systems known as T-tubules and sarcoplasmic reticulum; SERCA pumps are located in RS membranes.
At rest, Ca^{2+} ions are sequestered in RS lumen. When an action potential goes through sarcolemma and reaches T-tubules, Ca^{2+} ions are released in the cytosol. Following an action potential, the DHPRs (dihydipyridine receptors) voltage-depenent calcium-channels on the membrane of TT, undergoes a conformational change. This change is transmitted, with a mechanism not yet fully clarified, to the calcium release channels RyRs (ryanodine receptors) on SR membranes. The opening of RyRs permits calcium flux from the lumen of SR to the cytoplasm, raising cytoplasm calcium concentration. When $[Ca^{2+}]$ increases, calcium binds troponins' complex, particularly TnC; this bond generates a conformational change in which tropomyosin rotates and exposes calcium's binding sites on actin. This bond in turn permits the dissociation of Pi + ADP from myosin heads and generates what is known as powerstroke. During the powerstroke, the previously cited slip event takes place. At this point, when ATP binds myosin heads, the initial condition is restored, and the crossed-bridges cycle can start over. What we just said is well shown in the picture below.



2.3. Structure and localization of the SERCA pump

By a structural point of view, SERCA pumps reveal a globular lobe that protrudes into the cytosol connecting with the SR membrane [1]. As viewable in UniProtKB server (UniProtKB AC: O14983, AC: Q0VCY0, AC: Q642Z0) SERCA's structure is almost identical in human and bovine species, and in zebrafish. This data is interesting in relations to the studies that we will consider ahead in this work. On the same database another important information can be learn: SERCA pumps are located in Endoplasmic or in Sarcoplasmic reticulum membrane, from this their denomination. In the image below a schematic diagram of the structure of SERCA is shown (Watson H., 2015)[2].



2.4. SERCA pump isofroms

Three different genes encode the SERCA pump family, that are highly conserved but located on different chromosomes (Periasamy and Kalyanasundaram, 2007)[3]. From these genes have been identified different isoforms SERCA1, 2 and 3. These isoforms show specificity both at tissue and at developmental level. Even though different isoforms are encoded from different genes, the structure of SERCA pumps is highly conserved, with a similarity percentage 84%-75%. The three cited isoforms are distributed in mammalian tissues as follows. SERCA1 is found only in skeletal fast-twitch muscle, in more detail SERCA 1a is the adult form, while SERCA 1b is the neonatal one. SERCA 2b is an ubiquitous isoform expressed in all cell types, at the same time SERCA 2a is mainly present in skeletal slow-twitch muscles and in cardiac muscle. SERCA 3, is rarely found in muscle cells, but it is universally expressed in non-muscle cells.

In this work we are going to describe a muscular pathology of human species named Brody disease, caused by mutations in SERCA1 gene.

Moreover, the human disease has been compared to pathologies affecting SERCA1 pump, described in cattle and zebrafish. Surprisingly, in these two

species a condition similar to Brody disease for phenotype and gene defects has been described.

Due to their significance, SERCA pumps' activity must be strictly regulated, this role is managed by endogenous small molecular weight proteins present both in skeletal and in cardiac muscle. The two main molecules involved in SERCA's regulation are phospholamban (PLN) and sacolipin (SLN). PLN ad its mechanisms are well known, particularly in relation to SERCA 2 pumps and slow-twitch muscle, whereas SLN's mechanisms are only beginning to be understood, but it is known that it can regulate both SERCA 1 and 2 isoforms.

Summing up what said in this chapter, SERCA is a key regulator of calcium hemostasis, in fact it acts to transport calcium ions from the cytosol back to the sarcoplasmic reticulum following muscle contraction.

During the excitation-contraction events, cytosolic [Ca^{2+}] is increased for a few milliseconds, if it remains high longer than needed it could be detrimental to cellular homeostasis.

From now on, conditions involving SERCA pumps' disfunction will be discussed in this work, with the intent to compare different models and suggest a hypothetical therapeutic approach for human Brody disease.

3. Pathologies and characterization

3.1. A zebrafish behavioral mutant: *accordion*

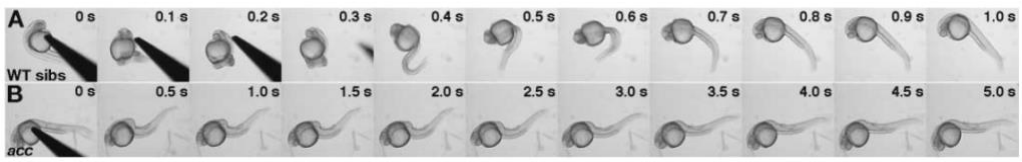
Since the end of the sixties, zebrafish has become an attractive model for the studies of motor development, due to its peculiar characteristics both as embryos and adults. Since SERCA1 protein is mainly expressed in muscles involved in movement, any damage or mutation of this protein results in a motor deficiency.

Several factors have made the zebrafish a smart vertebrate model for the study of human myopathies. Motor behaviours in zebrafish can be easily studied: three types of stereotyped movements that appear 36 hours post-fertilization (hpf), can be distinguished

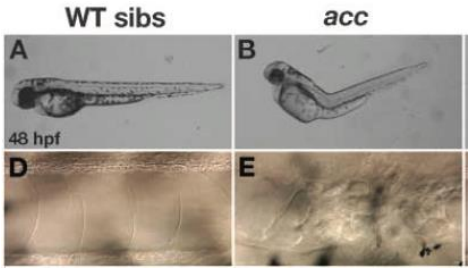
In order to study motor behaviours, we must distinguish the three types of stereotyped movements cited. At 17 hpf a repetitive spontaneous alternating coiling of the tail can be detected; after 21 hpf embryos start to respond to stimulation with coils stronger than those at 17 hpf. In the end, at 26 hpf, mechanosensory stimulation initiates swimming episodes. (Hirata et al., 2004)[4]

As for humans, when motoneurons are activated, they release acetylcholine in the neuromuscular junctions and initiate the muscle membrane's depolarization. Once the depolarization has started, it is detected by dependent L-type Ca^{2+} channels, and increased levels of cytosolic Ca^{2+} activates the cycle that gives life to the actual muscle contraction. Thanks to the pre-cited SERCA1 pumps,

cytosolic Ca^{2+} levels are rapidly decreased, to allow relaxation. Any defect in this pathway induces motor disorder pointed out by the following clinical signs: exercise-induced impairment of muscle relaxation, stiffening and cramps.
Because of the fact that zebrafish is a very interesting and useful study model, during the years a lot of behavioural mutants that showed abnormal movements were studied. A work developed in the early 2000s in the United States [4], grant us the ability to know the existence of *accordion*, a zebrafish behavioural mutant that exhibits apparently simultaneous contractions of trunk muscles on both sides of the embryo, resulting in the shortening of the trunk in response to touch. From now on we will refer to *accordion* mutation as "*acc.* phenotype". *Acc.* phenotype can originate due to different causes, one of these could be a muscle defect that increases the duration of contractions, without any defects in motoneurons' output. Thanks to the electrophysiologic studies carried out from Hirata's group [4], was shown that the output of CNS (central nervous system) and the function of NMJ (neuromuscular junction) are normal in *acc.*, however *acc.* trunk muscle stiffened for a while after active contraction, and the relaxation was five time slower than in wild-type animals. In order to focus on the muscle defect, was shown that overall morphology of the CNS was the same in wild-type and *acc*. embryos and rhythmic depolarizations were also similar, consistently with the fact that the output from the CNS and the response to the NMJ following mechanosensory stimulation is unmodified. Due to these considerations, the defect has to be found at muscular level.
Later was proved that the stiffness and the slowed relaxation are correlated to an impaired Ca^{2+} uptake, into the SR from the cytosol, after the contraction. As we previously noted, SERCA pumps are responsible for Ca^{2+} uptake and, as a confirm of the fact that *acc*. mutants have a weakened Ca^{2+} uptake from the cytosol, using a SERCA inhibitor generates in wild-type embryos the same symptoms as in *acc*.
In order to demonstrate that muscle relaxation is significantly slower in *acc*. mutants, three types of analyses were carried out: observation of the response to the touch, morphological observation and a DIC imaging.
Concerning the difference to the response to touch, the image below [4] well describes the time differences between wild-type and *acc*. mutants.



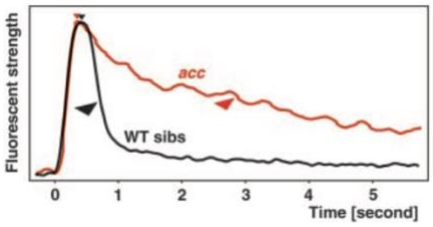
Three benchmarks are of interest for this analysis: active contraction, constant contraction and relaxation. However, even though the constant contraction period is significantly longer in *acc*. mutants, the main period of interest is the relaxation phase, that is five times longer in all *acc*. mutants, compared to WT siblings.



Morphological defects were also visible in *acc*. mutants, in fact their trunk is bent compared to that of wild-type siblings. However, these morphological defects are probably secondary due to mechanical stress and adaptation.

In the same image is visible a DIC (Differential Interference Contrast) imaging of the notochord, that shows how it is quite damaged in *acc*. mutants compared to WT animals.
At this point we have established that *acc*. mutants derive from a muscle defect and we have confronted them with wild-type siblings from different points of view.
In order to study the details of this muscle defect, measuring Ca^{2+} levels was fundamental. The Hirata group [4] did this injecting with Calcium Green-1 dextran, a fluorescent substance, in both *acc*. mutants and in wild-type embryos at 24 and 48 hpf. Fluorescence levels were observed by scanning with a confocal microscope during spontaneous coiling.

Results of this experiment showed how the time of Ca^{2+} increase, corresponding to the fluorescence's increase, was identical in mutants and WT, while the decay of Ca^{2+} was significantly slower in *acc*. mutants. This is well represented in the chart below [4].



At this point could be definitely affirmed that the clearance of Ca^{2+} from the cytosol is much slower in *acc*.'s muscles, this is coherent with a defect in SERCA1. Another confirm of this is given from the fact that a specific blocker of SERCA, thapsigargin, phenocopies the mutation in wild-type embryos.

Thanks to genomic and transcriptomic analyses, in *acc*. mutants, a mutation in the *ATP2A1* gene was found, this gene is known to encode for SERCA1. The Hirata group [4] gave us a confirm of this relation in zebrafish by rescuing mRNA into mutant embryos and by phenocopying the mutation. *ATP2A1* gene was mapped, and a point mutation in SERCA1 was found in all three alleles of *acc*. These three mutations that were found, were all locate in highly conserved α-helix domains that we cited in "chapter 1.2" of this work, these domains are M5, M2 and M7. Most mutations were found in M5 and result in a disruption or reduction of SERCA1 function, demonstrating the essential role of M5 domain. Mutation in M2 were also found to be possible, however these do not affect SERCA1 function itself but its regulation by sarcolipin, as previously said M2 regulate sarcolipin's binding. For what concerns M7, a mutation has been found so it surely exists, but nowadays it is not well known its function and the importance of this mutation.
At this moment is legit to affirm that another proof of the fact that the defect in *acc*. mutants is related to a muscle defect, is given from the fact that no expression of *ATP2A1* was observed at any level of the central nervous system.
At this point of our work, we have characterized the existence of a zebrafish behavioural mutant, known as *accordion*, and we have defined its peculiarities. Now we are going to deal with a similar condition that has been observed in Chianina cattle.

3.2. Chianina cattle muscular disorder: congenital pseudomyotonia (PMT)

In 2008 a congenital muscular disorder has been found in Chianina cattle, this condition is characterized by muscle contracture that prevents animals from performing muscular activities. Generally, animals experience this situation when stimulated to move faster than a slow pace walk, after transport stress or when startled; the stiffness disappears after a few minutes and the animal returns to a normal condition. One of the first studies that dealt with this condition defined it as "congenital pseudomyotonia" or PMT (Testoni et al., 2008)[5].
Nowadays, thanks to DNA sequencing techniques, the defect underlying Chianina PMT is fully characterized, a point mutation in exon 6 of the *ATP2A1* is held responsible. This mutation replaces an Arg at position 164 His, from now on we will refer to this mutation as R164H. An interesting study explored the biochemical characterization of activity and content of the mutated SERCA1 extracted from pathological muscles, in order to deepen our knowledge of this pathology (Sacchetto R. et al., 2009)[6].
All the analyses done in this study were done by confronting four Chianina animals PMT-affected and four normal Chianina animals.

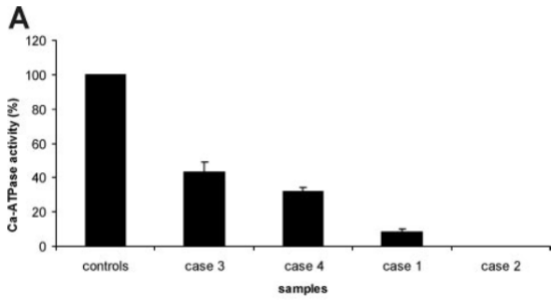
As the previously cited study [6] showed, the first difference between PMT-affected muscles and normal muscles could be noticed doing histological and

immunohistochemical analysis of muscle biopsies at rest and post exercise. While at rest and at slow pace no contractions were visible, post exercise - in affected Chianina - dark giant fibres with large areas of necrosis were visible. The degree of muscle damage is different in each animal.
Shifting on a biochemical level, Sacchetto's group was interested in investigating the effect of the previously mentioned point mutation of *ATP2A1* on the levels of expression of SERCA1 in affected muscles. These analyses were done thanks to the use of a monoclonal antibody to SERCA1 isoform. Results, considered on equal SR microsomal fractions from affected animals and by comparison from control muscles, showed a reduction of SERCA1 protein expression in all pathological sample examined. This was confirmed also using a different monoclonal antibody or a polyclonal antibody. In addition, the comparison showed a reduction of immunoreactivity to SERCA1 in PMT-affected muscles.
Another important information that this study [6] gave us is that the decrease in the content of SERCA1 is not counterbalanced by a corresponding increase of the other isoform, SERCA2. To support this was demonstrated that no difference exists in SERCA2 between normal and affected animals. In conclusion, all these results demonstrate that the decrease in immunoreactivity observed in immunofluorescence is due to a selective reduction of levels of expression of SERCA1 protein.
At this point a real-time PCR analysis was performed to question the possible relation between reduced content of SERCA1 and reduced transcriptional activity of *ATP2A1*. This RT-PCR pointed out no differences in mRNA expression levels in pathological samples and controls.

This last finding was investigated in a more recent study (Bianchini E. et al., 2014)[7], indeed the fact that in cattle SERCA1 mRNA levels in diseased muscle are normal while protein levels are markedly reduced suggested that the R164H mutation could cause SERCA1 misfolding and accelerate removal by the ubiquitin-proteasome system (UPS) or the autophagic-lysosomal pathway. To deepen this hypothesis, two suitable cellular models were developed: WT-HEK293 and R164H-HEK293. Once developed, these models were both incubated with a proteasome inhibitor, MG132, and it was shown that the expression levels of the SERCA1 mutant protein were significantly increased in MG132 treated cells. Two other specific inhibitors were used, and the results all together supported the hypothesis that SERCA1 mutant protein is selectively degraded by the UPS.
Using a combined approach involving a heterologous HEK293 cellular model overexpressing the mutant of SERCA1 and biopsies from PMT-affected cattle, was confirmed the hypothesis that the reduction of mutated SERCA1 protein was due to degradation by the ubiquitin-proteasome pathway. The most likely reason for this degradation by UPS is that the amino acid substitution leads to a

misfolded protein. These information permit us to classify cattle PMT into the family of unfolded protein diseases. Later on, in this work, we will discuss how the information given by Bianchini and her group [7] could be useful for an hypothetical therapeutic approach.

The last analysis that was done by Sacchetto and her group [6], was a measurement of Ca^{2+}-ATPase activity both in control animals and in PMT-affected Chianina. As well shown in the chart below, SERCA1's activity is significantly lower in affected animals than in healthy ones; the percentage of Ca^{2+}-ATPase activity is very subjective from case to case.



In conclusion nowadays we know that congenital pseudomyotonia in Chianina cattle is a condition of delayed muscle relaxation due to a prolonged elevation in cytoplasmic free Ca^{2+} concentration, and that a missense mutation in exon 6 of *ATP2A1* gene is the SERCA1's genetic defect that underlies Chianina-PMT.

Is interesting to notice that another similar condition exists, and it is known as Belgian Blue cattle congenital muscular dystony. The difference is that in Belgian Blue, the mutation is located within the nucleotide-binding domain, while in Chianina the mutation is in the A domain. In addition, affected Belgian Blue cattle face respiratory complications after a few weeks and die, while PMT in Chianina cattle is not life-threatening.

After affirming the existence of accordion and defined its peculiarities, we have examined the existence of a similar condition, known as PMT, in Chainina cattle. Now we are going to discover a human pathology that has the same symptoms as those observed until now.

3.3. Human's Brody disease

In 1969 Brody described, for the first time in history, a case of pronounced exercise induced muscle stiffness. Almost 20 years later, in 1986, Karpati discovered that SERCA1 protein deficiency had to be held responsible for the arising of this phenotype. At last, in 1996, the causative gene was found to be *ATP2A1*. Nowadays we know that Brody disease is a rare autosomal myopathy that is caused by mutations in the *ATP2A1* gene encoding the SERCA1 protein

and leading to exercise-induced muscle stiffness, sometimes symptoms as myalgia and muscle cramps can be present too. Because SERCA1 is expressed exclusively in fast-twitch skeletal muscles, stiffness is induced by phasic exercise and not by tonic activity.
In history only 18 patients have been reported with Brody disease, a recent study (Molenaar J.P. et al., 2020)[8] found out 22 new cases and tried to do an observational study in order to know more about this condition.
Investigations about symptoms permitted us to know that onset begins in childhood, but over time no or mild progression was observed. In all examined patients exercise-induced muscle stiffness was reported, and the muscle groups principally involved were lower and upper limbs and eyelids. Sometimes myalgia was described as a symptom too, and increasing of symptoms' intensity was reported after exposure to cold temperatures. However, impact on daily life is not very debilitating, the main features are the inability to run, difficulties when practicing sports and struggling to climb multiple stairs quickly.
In addition to this physical examination, histology was investigated as well and we gained the information that most patients displayed atrophy of type II fibres (76%), increased number of internal nuclei (82%) and high increase in muscle fibre size (92%).
Another crucial analysis done by Molenaar and his group [8] was the one about SERCA activity. Various techniques were used to investigate Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase activity, and in conclusion all the evaluations showed a decreased activity of 50-80%. Immunoblot and western blot analyses were performed on SERCA1 as well, and they all showed a reduced presence or absence of SERCA1 protein. On the other hand, immunohistochemistry was not very useful, in fact the results were very different among patients. Genetic analyses were interesting as well, as they granted us to know 33 different mutations of *ATP2A1* gene.
In last analysis, EMG was characterized by the presence of silent contractures and absence of myotonic discharges.
Even though little is known about Brody disease, this study [8] permits to classify it as a distinct myopathy in the broader field of calcium-related myopathies.
Another interesting aspect that was pointed out by Molenaar's group, was the fact that Brody disease's symptoms can be confused with a non-dystrophic myotonia. What can be emphasized to distinguish them, is that Brody disease only involves fast-twitch muscle fibres leading to selective muscle stiffness whit short, repetitive and explosive movements. In addition, a myotonic disorder will demonstrate myotonic discharges at myography, that we said are absent in Brody disease. What is also absent in Brody disease, but is frequently present in diseases like McArdle disease, are high creatine kinase levels and episodes of rhabdomyolysis.

What this study [8] suggests doing, when a case of Brody disease is suspected, is a specific sequencing of the *ATP2A1* gene, or a NextGen sequencing including *ATP2A1*. At this point, if an *ATP2A1* mutation is found, a muscle biopsy is recommended with a subsequent measurement of SERCA1 activity and a western blot analysis of SERCA1 to confirm the pathogenicity, and then diagnose Brody disease.
In conclusion, Brody disease (BD) is a "rare" genetic disorder due to defects in *SERCA1* gene characterized by exercise-induced muscle stiffness and impairment of relaxation. At present, neither mouse model [4] nor specific therapy [3] exist for Brody disease. Although mouse is undoubtedly the preferred animal model for preclinical evaluation of new therapeutic molecules, sometimes it fails to mirror the disease phenotype, resulting unsuitable to study certain human pathologies. This is the case of human Brody disease. As an example, wide differences in fibers composition of diaphragm muscle has been described between mice and large mammals, including humans. The impossibility to use mice as animal models for BD is going to be explained in the next chapter.

At this point of our work, we have described the existence of three conditions, in zebrafish, Chianina cattle and humans, that display very similar symptoms and that are dependent on a malfunction of SERCA1 pump and on a mutation of *ATP2A1* gene.
In the next chapter we are going to compare these conditions and to explain how *accordion* mutant and PMT can be models of interest to explore the condition of which we know less: human Brody disease.

4. Comparisons and animal models for human disease

4.1. Zebrafish *accordion* as a model for Brody disease

As we previously said, accordion is a behavioural mutant with a muscle relaxation defect due to a mutation in the ATPase Ca^{2+} pump SERCA1[4]. At this point we also know that impairment of Ca^{2+} regulation by SERCA1 mutation in human causes Brody disease, a rare inherited disorder of skeletal muscle function. Analyses done by Hirata's group pointed out how a mutation in the *ATP2A1* gene is responsible for *accordion*'s mutation. As we cited in chapter 2.3, mutations in *ATP2A1* are also responsible for human Brody disease. These two considerations make the *acc*. mutant an attractive animal model for Brody disease, about which little is still known.
In the last few years, nine mutations were isolated generating the *acc*. phenotype, all of which showed an autosomal recessive inheritance pattern, the same pattern that is found in most pathological cases of Brody disease. The consequences that these mutations have on zebrafish are muscle stiffness and a relaxation phase five time slower than normal. The main symptom of human

Brody disease is exercise-induced muscle stiffness. These considerations altogether permit us to affirm that zebrafish *acc*. mutants and Brody-affected patients share the most crucial feature of aberrantly slow relaxation of muscles because of a much slower re-uptake of Ca^{2+} from the muscle cytosol to the SR. [4]
In chapter 2.1 we affirmed that, since the end of the sixties, zebrafish has become an attractive model for the studies of motor development, due to their characteristics. Generally, *acc*. mutants die by day 10, however they have a fast development and large accessibility that permit molecular, genetic, pharmacological and physiological analyses. These characteristics make *accordion* mutants an interesting animal model not only to know more about Brody disease, but also to screen hypothetical drugs to relieve the muscle defect in vivo.
In addition, a positive aspect about using zebrafish mutants as animal models for Brody disease is given by the fact that analyses can be done on a large number of *accordion* mutants. This last consideration is very useful considering that, not only Brody disease is a rare pathology, but also that the largest study existing on humans is the one done by Molenaar's group [8], and it only questions 40 patients.

What is interesting to notice is that, while zebrafish represents an interesting animal model for human Brody disease, the same can not be said for mice, that usually are used as animal models due to their anatomical and genomic similarities with humans. What has been found is that SERCA1-null mice are defective in diaphragm function and died soon after birth, this condition make them not attractive for our studies.

4.2 Chianina cattle PMT as a model for Brody disease

In the previous chapter we not only underlined the muscle defect in zebrafish mutants, but also discussed about the existence of PMT, a Chianina cattle muscle disfunction characterized by muscle stiffness.
As we considered, a similar pathology exists in humans and the mechanism underlying it was found to be an abnormally low rate of Ca^{2+} uptake in the SR, resulting either from a reduced expression of SERCA1 protein or from a reduced activity of the normally expressed enzyme [6].
Similar clinical phenotypes were noticeable in Brody's disease affected patients and in PMT affected Chianina.

The biochemical studies done by Sacchetto's group on muscles of PMT-affected Chianina cattle, permitted to underline some similarities between PMT and Brody disease. As in human Brody disease, it was found that the Ca^{2+}-ATPase activity of SR membranes isolated from PMT affected cattle muscles, was markedly reduced, without any significant change in Ca^{2+}-dependency [6].

These data strongly correlate with the previously cited decrease in SR SERCA1 protein level in cattle pathological muscles. In addition, these considerations also correlate with measurements of Ca^{2+}-ATPase activity obtained from human microsomal SR fraction from Brody patients.
In the late 80ies, Karpati et al [9], used electron microscopy techniques to show that in muscle biopsies from patients affected by Brody disease, triads as well as the SR longitudinal component were normal in size and distribution. However, in pathological samples, an additional isoform of CS was found. These results well correlated with those found out by Sacchetto's group in SR muscle fractions from affected animals.

Previously, in chapter 2.2 of this work, we said that another condition, similar to PMT, exists and it is known as Belgian Blue cattle congenital muscular dystony; so, can we overall say that both congenital PMT in Chianina cattle and congenital muscular dystony in Belgian Blue cattle resembles human Brody disease and represent a suitable animal model? No, we can not. As for mice, cited in chapter 3.1, affected Belgian Blue cattle do not survive more that few weeks because of respiratory complications. On the other hand, in Chianina cattle as in humans the pathology is not life-threatening and respiratory failure is not a clinical feature, this suggests that Chianina cattle PMT might be the true counterpart of human Brody disease and that this bovine species might be used as a suitable animal model [6].

4.3 Overall conclusion about these comparisons
In conclusion, what is pointed out in this chapter is that, even though little is still known about human Brody disease, multiples counterparts exist and hypothetically permit new studies and discoveries.
The first animal model suitable for Brody's disease is represented by *accordion*, a zebrafish mutant. *Acc*. mutants were found to be useful due to their symptoms, similar to those showed by Brody-affected patients, and to the fact they have a fast development and large accessibility that permit molecular, genetic, pharmacological and physiological analyses. In addition, we said that studies on *acc*. mutants permit us to analyse a large number of animals.
This last aspect is not covered by the second relevant animal model that we could find: Chianina cattle PMT-affected. Congenital PMT has been demonstrated to have a lot of features similar to human Brody disease, both on symptomatic and biochemical levels; these data altogether make Chianina cattle a suitable animal model as well.
On the contrary it was demonstrated that some animals and some pathologies are not suitable as models form Brody disease, even though we could say so at first impact. First, mice- often used as animal models- are not useful in this situation, this is because those affected die soon after birth and no relevant analyses can be done. We face a similar problem with Belgian Blue cattle

affected by a congenital muscular dystony, in fact this pathology generates respiratory defects that lead to death.

At this point of our work, we have not only described the existence of three similar conditions, in zebrafish, Chianina cattle and humans, but also compared them in order to underline how we can improve the studies on the pathology that is the closest to us: human Brody disease.
In the next chapter we will provide a hypothetical therapeutic approach against Brody disease that starts with the inhibition of ubiquitin proteasome system.

5 Possible therapeutic ideas

5.1 UPS involvement in Chianina cattle PMT: considerations, analyses and results

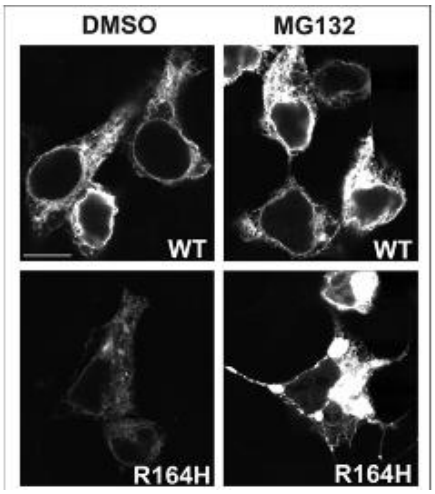
As we learned from the previously cited studies and thanks to what said in chapter 2 of this work, nowadays we know that a missense mutation in *ATP2A1* gene causes an impairment of muscle relaxation induced by exercise in Chianina cattle, this impairment is known as PMT (Cattle "congenital pseudomyotonia). A study done in 2008 sequenced DNA of affected Chianina cattle in order to provide evidence of a missense mutation in exon 6 of *ATP2A1* gene, that we know encodes for SERCA isoform 1. This mutation replaces an *Arg* at position 164 by *His* (R164H), in a highly conserved region of SERCA1 protein. At the end, studies conducted between 2008 and 2009, concluded that the decrease in SR Ca^{2+}-ATPase activity found in affected animals was mainly due to reduction of SR SERCA1 protein content. The consequent reduction in pumping efficiency of SR is likely responsible for muscle stiffness.

In the previous chapter we said how *accordion* mutant and PMT affected Chianina cattle are interesting animal models to study human Brody disease. Particularly, the relevance of PMT affected Chianina cattle as animal models is given by the clinical phenotype similarity with human Brody disease. In fact, exercise-induced muscle stiffness and delayed muscle relaxation are observable both in Brody affected people and in PMT affected animals. In addition, both cattle congenital PMT and Brody disease are genetically heterogeneous. Thus, it is possible to affirm that Chianina cattle congenital PMT is a true counterpart of human Brody disease, making it a useful, although unconventional, animal model, also to study possible therapeutic approaches, that do not exists nowadays for Brody disease.

In a study done in 2014 by Elisa Bianchini and her group (Bianchini E., et al; 2014)[10], was observed that in cattle, SERCA1 mRNA levels in diseased muscles are normal, while protein levels are markedly reduced, suggesting that

the R164H mutation could cause SERCA1 misfolding and accelerated removal by either the ubiquitin-proteasome system (UPS) or the autophagic-lysosomal pathway.
In this study different kinds of analyses were done, starting with Mutagenesis, Transfection and treatment with UPS inhibitors, going on with Biopsies, Immuocytochemical analysis, Gel Electrophoresis and Immunoblotting, and concluding with Immunoprecipitation and Aequorin Ca^{2+} measurements.
During these analyses was necessary to develop a cellular model suitable for investigating the role of UPS, to do that HEK293 cells were transfected with cDNA encoding WT o R164H mutant SERCA1, these cellular lines are respectively known as WT-HEK293 and R164H-HEK293.
Immunofluorescence analyses showed that both in WT-HEK293 and R164H-HEK293, SERCA1 is correctly targeted to ER, but the expression level of R164H SERCA1 was much lower. These data were confirmed by Immunoblot analysis.
Because the interest was on the role of UPS, Bianchini's group incubated both WT-HEK293 and R164H-HERK293 with MG132, a well-known proteasome inhibitor.

As visible in the image below, cells expressing R164H SERCA1 mutant showed a striking increase of immunoreactivity of SERCA1 after treatment with MG132; an increase was visible also in WT-HEK293, but it was not as strong as in R164H-HEK293.



To confirm the hypothetical role of UPS, R164H-HEK293 and WT-HEK293 were incubated with other highly specific proteasome inhibitors, in these conditions the same results were registered.
At this point, Bianchini and her group, decided to study the degree of ubiquitination of the SERCA1 mutant and, knowing that MG132 inhibits UPS

downstream of the ubiquitination step, they hypothesized that treatment with MG132 should have allowed recovery of mutated SERCA1 in a polyubiquitinated form. To confirm this hypothesis both WT and R164H cells, treated or not wit MG132, were lysed in mild conditions to permit the subsequent immunoprecipitation assay. The results demonstrate very clearly that blockade of UPS activity in R164H-HEK293 causes accumulation of SERCA1 protein.
Further analyses confirmed two other key points related to the UPS system. First, measuring Ca^{2+}-ATPase activity, was confirmed thar R164H SERCA1 mutant treated with MG132 exhibited functional properties similar to those of WT SERCA1. Secondly, cytosolic Ca^{2+} transient was monitored in cells incubated with MG132 in order to demonstrate that the physiological Ca^{2+} homeostasis in cells expressing mutated SERCA1 can be restored by blocking the UPS.

5.2 Therapeutic approach against Brody disease taking advantage of the functional rescue of mutated SERCA1

Overall, this study started from the consideration that the missense mutation in *ATP2A1* gene found responsible for Chianina cattle PMT does not affect the gene transcription itself, and so it was hypothesized that the reduction of mutated SERCA1 protein was due to degradation by the UPS. Altogether the results of this study show that the R164H SERCA1 is degraded by UPS, and that the likely reason for this is that the amino acid substitution leads to a misfolded protein and that the UPS is thought to be mainly involved in the removal of misfolded mutants [10].

In conclusion the information given us from this study could be really useful, because at present no specific therapy exists. The findings underlined in this chapter showed us that proteasomal inhibition functionally rescues the mutated SERCA1 in a cell model as well as in muscle fibres from PMT affected animals. These results are very important because they open a door for a possible therapeutical approach in vivo.

6 Final considerations

Even though this work is centered on SERCA pumps malfunctions and on the pathology correlated, a wide excursus has been done starting from general knowledge about SERCA pumps and ending with hypothetical therapeutic approaches.
At the beginning a complete description of the SarcoEndoplasmic Reticulum Calcium ATPase pump has been done. This description focused not only on the structure of SERCA pumps, but also on their isoforms, localization and role;

this last aspect can be considered as the most important to learn when approaching to this work, in fact the whole thesis work is focused on SERCA pumps malfunctions, but it is fundamental to learn the normal physiological function if we really want to comprehend malfunctions and pathologies. What was also very important to remember in this context is the general physiology of muscle contraction and the role of calcium in it, in fact only understanding its functioning and calcium importance it is possible to understand why SERCA pumps related pathologies are so impactful.

Stated the generals, an excursus on pathologies linked to SERCA1 malfunctions has been done. In particular three models were represented in this work: a zebrafish behavioral mutant known as *Accordion* mutant, Chianina cattle PMT affected and humans affected with Brody disease. The main feature that connects all these models is muscle stiffness post-exercise. As said, molecular and biochemical studies on all these models showed a defect on SERCA1.

Why learning about *Accordion* mutant and congenital pseutomyotonia was so important is demonstrated in chapter 3 of this thesis work. Both zebrafish mutant and PMT affected Chianina cattle resulted to be very useful animal models to study human Brody disease, which is very rare and little is known about.

The usefulness of *Acc*. mutant is given by three main aspects, first zebrafish have a fast development that permits to speed up the analyses, at the same time the studies can be not only fast but also on a large scale, thanks to the wide number of zebrafish accessible in laboratories. Last but not least, a large number of analyses can be done easily on zebrafish, being them molecular, genetic, pharmacological or physiological.

At the same time, the studies on PMT affected Chianina cattle are very interesting because of the muscle similarities between cattle and humans; in addition, the biochemical studies showed how similar clinical phenotypes in these two models were linked to biochemical similarities connected to SERCA1 activity.

What makes these two animal models even more important for studying the human condition is the fact that mice, a common animal model when we want to deepen human pathologies, is totally useless in this case. It has been demonstrated SERCA1-null mice are defective in diaphragm function and died soon after birth, which makes them worthless for studies.

The final part of this work is centered on a hypothetical therapeutic approach, in fact not only Brody disease is very rare, but nowadays a therapy for it does not exist. The studies on PMT made possible to investigate the role of UPS in this pathology, and it was demonstrated that the physiological Ca^{2+} homeostasis in cells expressing mutated SERCA1 can be restored by blocking the UPS. This knowledge opened the hypothesis of a therapeutic approach against Brody disease taking advantage of the functional rescue of mutated SERCA1; this

approach could be revolutionary, considering the life impact of Brody condition.

In conclusion this work could be useful for further studies because it sums up almost everything that there is to know about SERCA pumps disfunctions and on the pathologies related. In addition, it gives an initial overview for presenting the physiological situation to everyone wo would like to approach to this theme. The end of this paper, even though does not give a sure therapy, gives us the understanding that steps are being done in this field and the hope that we are not far from a therapy that would improve the lives of Brody affected patients.

7. Bibliography

[1] Xu, H., Van Remmen, H., 2021. The SarcoEndoplasmic Reticulum Calcium ATPase (SERCA) pump: a potential target for intervention in aging and skeletal muscle pathologies. Skeletal Muscle 11, 25. https://doi.org/10.1186/s13395-021-00280-7

[2] Watson, H., 2015. Biological membranes. Essays in Biochemistry 59, 43–69. https://doi.org/10.1042/bse0590043

[3] Periasamy, M., Kalyanasundaram, A., 2007. SERCA pump isoforms: Their role in calcium transport and disease. Muscle Nerve 35, 430–442. https://doi.org/10.1002/mus.20745

[4] Hirata, H., Saint-Amant, L., Waterbury, J., Cui, W., Zhou, W., Li, Q., Goldman, D., Granato, M., Kuwada, J.Y., 2004. *accordion* , a zebrafish behavioral mutant, has a muscle relaxation defect due to a mutation in the ATPase Ca2+ pump SERCA1. Development 131, 5457–5468. https://doi.org/10.1242/dev.01410

[5] Testoni S, Boni P, Gentile A: Congenital pseudomyotonia in Chianina cattle. Vet Rec 2008, 163:252

[6] Sacchetto, R., Testoni, S., Gentile, A., Damiani, E., Rossi, M., Liguori, R., Drögemüller, C., Mascarello, F., 2009. A Defective SERCA1 Protein Is Responsible for Congenital Pseudomyotonia in Chianina Cattle. The American Journal of Pathology 174, 565–573. https://doi.org/10.2353/ajpath.2009.080659

[7] Bianchini, E., Testoni, S., Gentile, A., Calì, T., Ottolini, D., Villa, A., Brini, M., Betto, R., Mascarello, F., Nissen, P., Sandonà, D., Sacchetto, R., 2014. Inhibition of Ubiquitin Proteasome System Rescues the Defective Sarco(endo)plasmic Reticulum Ca2+-ATPase (SERCA1) Protein Causing Chianina Cattle Pseudomyotonia. Journal of Biological Chemistry 289, 33073–33082. https://doi.org/10.1074/jbc.M114.576157

[8] Molenaar, J.P., Verhoeven, J.I., Rodenburg, R.J., Kamsteeg, E.J., Erasmus, C.E., Vicart, S., Behin, A., Bassez, G., Magot, A., Péréon, Y., Brandom, B.W., Guglielmi, V., Vattemi, G., Chevessier, F., Mathieu, J., Franques, J., Suetterlin, K., Hanna, M.G., Guyant-Marechal, L., Snoeck, M.M., Roberts, M.E., Kuntzer, T., Fernandez-Torron, R., Martínez-Arroyo, A., Seeger, J., Kusters, B., Treves, S., van Engelen, B.G., Eymard, B., Voermans, N.C., Sternberg, D., 2020. Clinical, morphological and genetic characterization of Brody disease: an international study of 40 patients. Brain 143, 452–466. https://doi.org/10.1093/brain/awz410

[9] Karpati, G., Charuk, J., Carpenter, S., Jablecki, C., Holland, P., 1986. Myopathy caused by a deficiency of Ca2+-adenosine triphosphatase in sarcoplasmic reticulum (Brody's disease). Ann Neurol. 20, 38–49. https://doi.org/10.1002/ana.410200108

[10] Bianchini, E., Testoni, S., Gentile, A., Calì, T., Ottolini, D., Villa, A., Brini, M., Betto, R., Mascarello, F., Nissen, P., Sandonà, D., Sacchetto, R., 2014. Inhibition of Ubiquitin Proteasome System Rescues the Defective Sarco(endo)plasmic Reticulum Ca2+-ATPase (SERCA1) Protein Causing Chianina Cattle Pseudomyotonia. Journal of Biological Chemistry 289, 33073–33082. https://doi.org/10.1074/jbc.M114.576157