

*Original article* ■

# Unfolded Protein Response and Triad Formation in Skeletal Muscles of Catecholaminergic Polymorphic Ventricular Tachycardia Mouse

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## SUMMARY

Isoform 2 of calsequestrin (CASQ2) is the main calcium-binding protein of sarcoplasmic reticulum (SR), expressed both in cardiac and in skeletal muscles. CASQ2 acts as an SR calcium ( $\text{Ca}^{2+}$ ) sensor and regulates SR  $\text{Ca}^{2+}$  release via interactions with triadin, junctin, and the ryanodine receptor. Various mutations of the *csq2* gene lead to altered  $\text{Ca}^{2+}$  release and contractile dysfunction contributing to the development of arrhythmias and sudden cardiac death in young individuals affected by catecholaminergic polymorphic ventricular tachycardia (CPVT).

Recently, a transgenic mouse carrying one of the identified CASQ2 point-mutations (R33Q) associated to CPVT was developed and a drastic reduction of the mutated protein was observed. Following a biomolecular approach, several analysis were performed using different antibody treatments in order to identify when the reduction of CASQ2 begins in skeletal muscles, unveil the mechanism involved in the reduction of CASQ2 in slow-twitch and fast twitch muscles and verify if other proteins are affected by the presence of the mutated protein.

Mutated CASQ2 decreased soon after birth. Up-regulation of proteins associated to the unfolded protein response (UPR) was also observed. Important proteins in skeletal muscle triads formation were analyzed and increased protein levels were observed in adult knock-in CASQ2-R33Q/R33Q mice.

Probably, R33Q mutation induced the decrease of CASQ2 by activation of the UPR and subsequently degradation through proteasome.

**Key words:** calsequestrin, endoplasmic reticulum associated degradation, unfolded protein response, triads, chaperones

## INTRODUCTION

Sudden cardiac death causes approximately 300.000 deaths each year in Europe and the USA; ventricular fibrillation is the underlying mechanism to most of diseases that lead to sudden cardiac death. The identification of mechanisms causing life-threatening arrhythmias is a major priority in the biomedical field (1, 2). Given the crucial role of  $\text{Ca}^{2+}$  in focal arrhythmias generation, the mechanisms responsible for  $\text{Ca}^{2+}$  homeostasis in SR of cardiomyocytes has acquired great importance in cardiac pathophysiology. The discovery of SR proteins that play a key role in these mechanisms, ryanodine receptor (RyR) and CASQ, in inherited arrhythmia syndromes, such as CPVT, emphasizes the link between changes in intracellular  $\text{Ca}^{2+}$  homeostasis and the arrhythmogenesis (3, 4). The CPVT occurs at a young age as a result of exercise or emotional stress and it is characterized by recurrent syncope, convulsions, cardiovascular collapse and can lead to sudden death (4). This disease is characterized by a normal baseline ECG showing a  $180^\circ$  axis rotation of the QRS complex (typical of bidirectional ventricular tachycardia); it is also characterized by the absence of structural abnormalities of the heart (5). Although it is a rare inherited disease, it is a clinical model of triggered activity (TA) mediated by adrenergic stimuli and consequently is a very useful tool in the investigation of some of the pathophysiological unsolved arrhythmogenesis aspects. The CASQ2 homozygous mutations (6) explain the 3% of CPVT cases, while about 50% of patients have mutations in the RyR2 (7). Song (8) and colleagues have demonstrated the existence of common pathophysiological mechanisms between CPVT caused by alterations in RyR2 and/or CASQ2. Experimental data have confirmed that common mechanisms underlying the ventricular arrhythmias are the after-depolarizations delayed (DADs) and triggered activity TA caused by spontaneous release of calcium during adrenergic stress (9). In order to bring experimental data close to clinical ones, the development of animal experimental models is extremely important and it must be a phenotypic copy of clinical disease. This "challenge" is particularly demanding in arrhythmogenic diseases, because of the large differences between humans and mice in ion channels profiles and in action potentials duration. The CPVT proved to be an exception in comparison to other hereditary diseases, represented by transgenic mice, which have low analogy with human patient phenotype. Actually, knock-in (KI) RyR2-R4497 (10) and CASQ2-R33Q (11) models exist as autosomal dominant and recessive CPVT models, respectively. These mice show bidirectional and polymorphic ventricular tachycardia (consistent with the CPVT phenotype observed in patients) and develop DADs and TA after adrenergic stimulations. However, it is believed that these knock-in mice are not useful only and exclusively to investigate several important aspects of CPVT, but also for better understanding the  $\text{Ca}^{2+}$  release control and its role in

acquired arrhythmogenic conditions. They are also useful to test multiple approaches in order to develop new therapeutic strategies. The mechanism that associates the depolarization of muscle fiber plasma membrane followed by the release of  $\text{Ca}^{2+}$  from the SR, called coupling excitation-contraction (EC), depends on the plasma membrane calcium channel L type, receptor for the dihydropyridine (DHPR), and the RyR receptor present on the membrane of SR terminal cisternae; both constitute the  $\text{Ca}^{2+}$  releasing units (CRU) (12). The DHPR is localized on the plasma membrane and its invaginations, the T tubules, and particularly is positioned in the junctions between plasma membrane and SR terminal cisternae. During the *plateau* phase of the cardiac action potential, a small amount of  $\text{Ca}^{2+}$  enters in cardiomyocytes via the DHPR causing the release of  $\text{Ca}^{2+}$  from the lumen of SR to the cytosol via RyR2. This process is called the release of calcium induced by calcium (ICRC) (13). In the relaxation phase  $\text{Ca}^{2+}$  is removed from the cytosol through SERCA pumps, which back calcium in the SR at the expense of ATP consumption. The cytosolic  $\text{Ca}^{2+}$  is removed during the diastolic phase not only by the action of SERCA pumps, but also from that of the sodium/calcium exchanger (NCX), positioned on the surface of the plasma membrane, which promotes the release of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  uptake. In this phase, the under threshold  $\text{Ca}^{2+}$  release causes the continuous entry of  $\text{Na}^+$ , which may increase the probability of DADs and cardiac arrhythmias events. The calsequestrin is the major  $\text{Ca}^{2+}$ -binding protein present in the junctional SR of skeletal and cardiac muscle fibers. In mammals, there are two CASQ isoforms encoded by different genes: the skeletal isoform, CASQ1 is expressed in fast and slow-twitch skeletal muscle, while the cardiac isoform, CASQ2, is expressed in both cardiac and skeletal muscles. The CASQ plays an important role as a buffer for  $\text{Ca}^{2+}$  and regulates the activities of RyR in relation to the concentration of  $\text{Ca}^{2+}$  present in the lumen of the SR (15). Furthermore, it is proposed to play an important role in SR junctions' development, together with Trd and Jct (16). It is present as linear polymer into the lumen of the SR terminal cisternae and is anchored to the RyR through two transmembrane proteins, the Trd and the Jct, forming the complex of  $\text{Ca}^{2+}$  release channel (14). Some of the mutations in the CASQ2 seem to affect the synthesis of the protein leading to its reduction or absence in the heart (17), while other mutations seem to induce the expression of defective proteins, which show altered ability to bind  $\text{Ca}^{2+}$  and/or perform their proper function (18). Based on previous experimental evidence, the working hypothesis are that CASQ2 mutations operate through at least two converging mechanisms which induce  $\text{Ca}^{2+}$  irregular transients and DADs (19, 20). CASQ2 mutation knock-in mouse (11) was characterized *in vitro* and *in vivo* and was demonstrated that it was an identical copy of the patient phenotype. Reduced CASQ2 and Trd protein levels were present, while the corresponding mRNAs levels remained

unchanged (11). All these experimental evidence implies probable post-transcriptional regulation mechanism implications. Among the possible mechanisms involved in this regulation exists protein degradation associated with the endoplasmic reticulum (ER), known as endoplasmic reticulum-associated degradation (ERAD), which is responsible for the ER lumen synthesized proteins degradation via the proteasome after the activation of an ER stress response. Various factors can activate an ER stress response. One of them is the presence of incorrect folded proteins (unfolded protein response, UPR). The first objective of this research was the age determination of the CASQ2 reduction beginning in skeletal muscles. The others were the determination of whether any other protein was affected by reduced levels of CASQ2 and the revelation of the mechanisms involved in the reduction of CASQ2-R33Q in skeletal muscles.

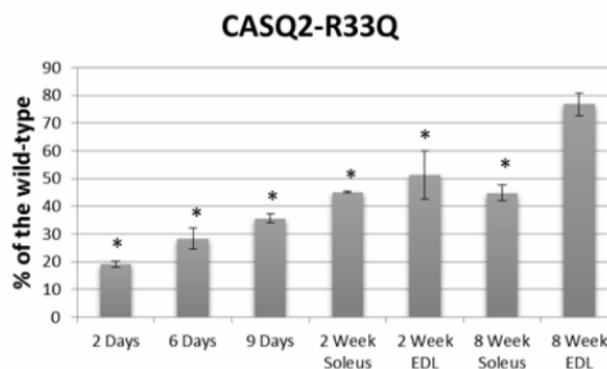
## MATERIALS AND METHODS

Molecular analysis were performed on skeletal muscle total homogenates of wt / wt and knock-in CASQ2-R33Q/R33Q mice at the University of Padova. All experimental protocols have been approved by Institutional Ethical Committees of the Universities of Padova and of Chieti. Mice were kept in accredited animal facilities and killed by anaesthetic euthanasia. Four legs for each genotype were used and only male mice were selected in order to perform these analysis. In these analyses some criteria were chosen in order to obtain completeness of postnatal development information and have a detailed picture of the events that were triggered by the presence of R33Q point mutation in CASQ2. Therefore, first an age that was closest possible to birth (2 days) was used, and then 1 week after birth. Nine days old mice were chosen for molecular analysis, because this age is an intermediate period time between the first and the second week. The period of two weeks after birth was considered as a critical time for the myocyte, because previous studies results (12) showed that at this phase, the optimal morphological characteristics appeared in the myocyte and those were responsible for the correct functioning of the skeletal muscle in adulthood. Week 8 was a good period to analyze adulthood as reported by Flucher and Franzini-Armstrong (12). The slow-twitch (soleus) and fast twitch muscles (*Extensor Digitorum Longus* - EDL) were taken from the 2 and 8 week old mice's well-developed legs. The samples were homogenized and the corresponding proteins were quantified using Lowry biochemical method. Subsequently, for each sample, 15-50 µg of protein were loaded on 7.5-10% SDS-polyacrylamide gels depending on the protein being investigated. The following dilutions and primary antibodies were used: polyclonal anti both CASQ1 /CASQ2 (1:1,000), polyclonal anti BiP (GRP78) (1:500), and polyclonal anti CRT (1:1000) were all from Affinity Bioreagents (Affinity Bioreagents, Golden, CO); polyclonal

anti GRP94 (1:1,000), monoclonal anti Cav-3 (1:1000), polyclonal anti JP-1 (1:1000) and JP-2 (1:1000) were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary anti-mouse, anti-goat or anti-rabbit (1:5,000) alkaline phosphatase-conjugated antibodies were from Sigma (Milan, Italy). After electroblotting on nitrocellulose paper, the equivalence and homogeneity of the loaded samples were verified by staining the nitrocellulose membrane with Ponceau Red. The immunodecorated bands were visualized by a ready-to-use, precipitating substrate system for alkaline phosphatase (BCIP/NBT liquid substrate system; Sigma). Images were obtained with an HP Scanjet scanner and Adobe Photoshop CS2 version 9.0. Densitometry was performed with Scion Image Software without modification of the images (raw images) to quantify protein band intensities. Data were expressed as means ±SE. Statistical analysis were performed using Origin 8.5 software. Student's *t*-test was used for comparisons between wt/wt and knock-in CASQ2-R33Q/R33Q data. Statistical significance was set at  $P < 0.05$ .

## RESULTS AND DISCUSSION

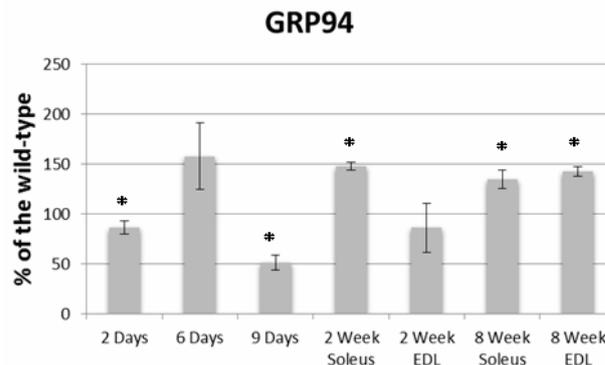
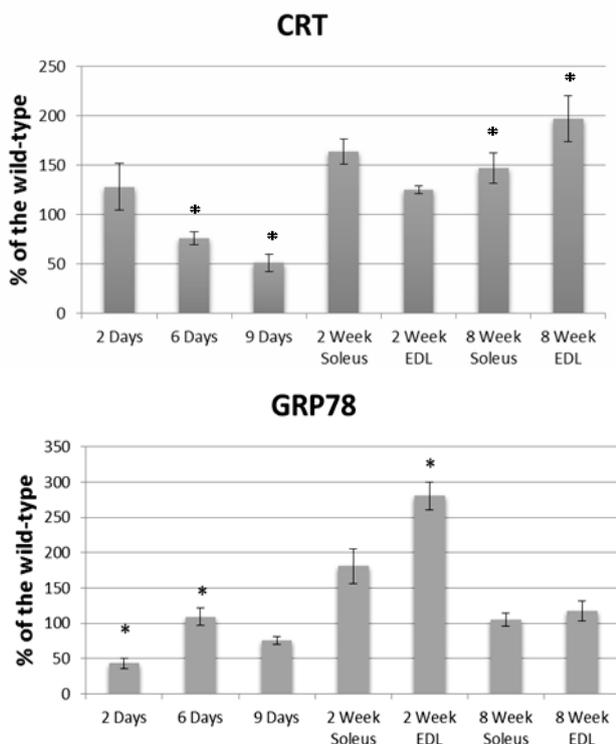
CASQ2-R33Q is a CASQ2 with a mutated amino acid in position 33, which did not seem to affect the secondary and tertiary conformations of the overall protein (20). CASQ2 is not present only in the heart, but it is present in the skeletal muscles, too. Skeletal muscles were analyzed in order to study the CASQ2 mutation effects in skeletal muscles. Thus, molecular analyses were performed on skeletal muscle total homogenates of wt/wt and knock-in CASQ2-R33Q/R33Q mice. As it is shown in Figure 1, the protein level of CASQ2-R33Q was not reduced, but rather it increased after birth. However, the knock-in mice CASQ2 protein levels were lower than wild type mice CASQ2 levels.



**Figure 1.** CASQ2 protein levels comparison between CASQ2-wt/wt and knock-in CASQ2-R33Q/R33Q mice of different ages. CASQ2 expression levels of homozygous knock-in mice are expressed in percent of wt mice CASQ2 levels. (\*)  $P < 0.05$  same age comparison between knock-in CASQ2-R33Q/R33Q and CASQ2-wt/wt mice protein levels.

One of the mechanisms which can explain the reduction of CASQ2-R33Q without affecting its mRNA content is a post-transcriptional control, as it happens for unfolded proteins. An imbalance between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery to handle this load is called ER stress, and it causes a series of responses. In eukaryotic cells, the majority of secreted proteins and transmembrane are synthesized and matured in the ER lumen. Proteins enter the ER as unfolded polypeptide chains. Incorrect folding of nascent proteins may occur in response to many environmental stresses or as a result of mutations that disrupt the proteins structure. The imbalance between the load of unfolded proteins that enter the ER and the ability of the cell *machinarium* handling this load is defined as ER stress, which triggers a series of responses known as UPR (21). When ER stress occurs, three ER transmembrane sensors are activated to initiate adaptive responses. These sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and the transcriptional factor activating transcription factor 6 (ATF6). Subsequently, there is an increase in the capacity of the ER to handle unfolded proteins, which is a longer term adaptation that entails transcriptional activation of UPR target genes, including those that function as part of the ER protein-folding machinery (ER chaperones), like calreticulin (CRT), glucose-regulate protein-78 (GRP78) and -94 (GRP94) (22).

It is thought that GRP94 plays an important role in incorrectly folded proteins recognizing and participates in these protein translocation from lumen to the cytosol (22). In order to explain CASQ2-R33Q reduction in skeletal muscles we analyzed ER chaperones protein expressions (Figure 2).

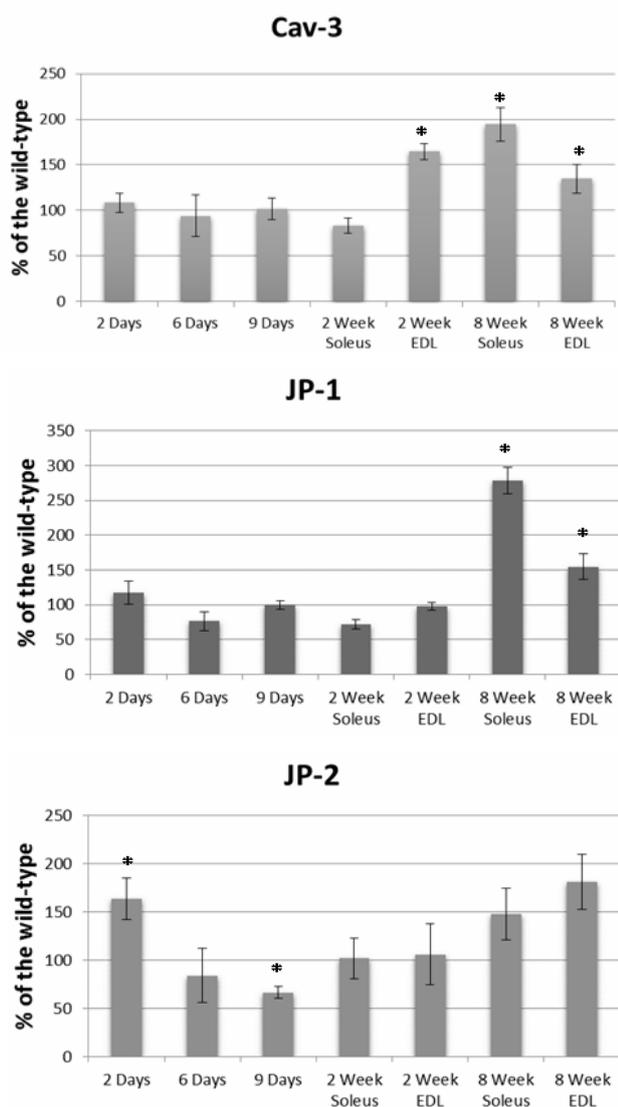


**Figure 2.** CRT, GRP78 and GRP94 protein levels comparisons between wt/wt and knock-in CASQ2-R33Q/R33Q mice of different ages. All analyzed chaperones expression levels of homozygous knock-in mice are expressed in percent of wt relative protein levels. (\*)  $P < 0.05$  same age comparison between knock-in CASQ2-R33Q/R33Q and CASQ2-wt/wt mice protein levels.

It emerged that each chaperonin expression profile was very different from the others, even if they reach the peak at 2 week old knock-in CASQ2-R33Q/R33Q mice. Calreticulin plays also an important role as  $Ca^{2+}$  buffer protein in ER (23). In fact, it was manifested by its expression profile in skeletal muscles of the hind paw of 2, 6, and 9 days old knock-in CASQ2-R33Q/R33Q mice. A CRT protein levels reduction was observed, which might be linked to the increased CASQ2 protein levels, as another important  $Ca^{2+}$  buffer in myocytes (Figure 2).

GRP78 peaked at two week, although it was statistically significant only in the EDL of the knock-in CASQ2-R33Q/R33Q mice. Both soleus and EDL expression profiles showed that GRP94 and CRT protein levels of knock-in CASQ2-R33Q/R33Q mice were (statistically significantly higher than those of wt/wt mice even after two week from birth. In fact, in the soleus and EDL of eight week old knock-in CASQ2-R33Q/R33Q mice were observed statistically significantly higher protein levels than those relative to the wild type mice soleus and EDL, respectively. Thus, GRP94 protein levels remained high in soleus and EDL even after two weeks from mice birth. One of the other objective of this research was the determination of whether any other protein was affected by reduced levels of CASQ2. Another important protein in ER is RyR which interact with CASQ in order to ensure the normal contractile function. For this reason, the study was extended to other proteins that interact with RyR in various development stages. Golini and colleagues (24) demonstrated that junctophilin 1 and 2 (JP-1 and JP-2) are parts of a protein complex in skeletal muscles. This protein complex is also composed of RyR1, caveolin 3 (Cav-3) and DHPR. In muscle tissues Cav-3 form the caveolae, which are flask-shaped invaginations localized in the sarcolemmal membrane of myocytes and serves as a platform for T tubule formation (25). The junctophilin proteins were identified as very active candidates

in junctional membrane complex (JMC) formation (26). They represent a class of highly conserved proteins which are localized in JMC and involved into endo/sarco-plasmic membrane anchoring to sarcolemma/T tubule (24). Yamazaki and colleagues (27) showed that triads were generated in skeletal muscles where JP-1 and JP-2 were expressed, while dyads are formed in the heart muscle where only JP-2 was expressed. Cav-3, JP-1 and JP-2 play an important role in the triad formation. Their expression was analyzed by comparing wt/wt and knock-in CASQ2-R33Q/R33Q mice data (Figure 3).



**Figure 3.** Cav-3, JP-1 and JP-2 protein levels comparisons between wt/wt and knock-in CASQ2-R33Q/R33Q mice of different ages. All analyzed chaperones expression levels of homozygous knock-in mice are expressed in percent of wt relative protein levels. (\*)  $P < 0.05$  same age comparison between knock-in CASQ2-R33Q/R33Q and CASQ2-wt/wt mice protein levels.

The skeletal muscle results of Cav-3 expression profile of knock-in CSQ2-R33Q/R33Q mice showed a statistically significant increase only in the EDL of two and eight week old mice in comparison with the wild-type mice. This enormous increase in Cav-3 protein levels of the knock-in CSQ2-R33Q/R33Q mice suggested a possible increase of triad number in adult skeletal muscles. A further confirmation was reported by the significant increase of soleus and EDL JP-1 levels in the eight week old knock-in CASQ2-R33Q/R33Q mice.

Besides the lack of a statistically significant increase of JP-2 protein levels in 2 and 8 week old knock-in CASQ2-R33Q/R33Q mice, there was a statistically significant reduction. In 9-day-old mice, it reached a lower protein level in comparison with wild-type mice. JP-1 and JP-2 experimental data were supported by Yamazaki and colleagues suggestion (27). They suggested that JP-1 protein expression was the most crucial event for the maturation of dyad into triad. In fact, a statistically significant increase of JP-2 protein levels was observed only 2 days after the birth of knock-in mice, which could be decisive for the formation of dyads in skeletal muscle.

## CONCLUSIONS

All ER chaperones expression profile results suggested a possible ERAD activation which could be responsible for the CASQ2-R33Q reduction in skeletal muscles. However, in order to confirm all previously suggestions, further analysis of these muscular tissues would be necessary. The use of the electron microscope could be very helpful.

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## ODGOVOR RAZVIJENOG PROTEINA I FORMIRANJE TRIJADA U SKELETNIM MIŠIĆIMA MIŠEVA SA KATEHOLAMINERGIČKOM POLIMORFNOM VENTRIKULARNOM TAHIKARDIJOM

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### Sažetak

Izoforma 2 kalsekvestrina (CSQ2) je glavni kalcijum-vezujući protein sarkoplazmatskog retikuluma (SR) i ispoljava se i u srčanom i u skeletnom mišiću. CSQ2 deluje kao SR kalcijumski sensor, koji reguliše oslobađanje Ca<sup>2+</sup> jona iz sarkoplazmatskog retikuluma putem interakcije sa triadinom, junktinom i rianodinskim receptorom. Različite mutacije csq2 gena dovode do promena u oslobađanju Ca<sup>2+</sup> i kontraktilne funkcije i na taj način doprinose razvoju aritmija i iznenadnoj srčanoj smrti mladih osoba koje boluju od kateholaminergičke polimorfne ventrikularne tahikardije (CPVT).

Nedavnim razvojem transgenetskih miševa koji su nosioci CASQ2 point mutacija (R33Q) udruženim sa CPVT-om, uočeno je drastično smanjenje nivoa mutiranog proteina. U skladu sa biomolekularnim pristupom, izvedeno je nekoliko analiza primenom tretmana različitim antitelima kako bi se identifikovao početak smanjenja CASQ2 u skeletnim mišićima, otkrio mehanizam uključen u smanjenje CASQ2 kod sporo- i brzo-kontrahujućih mišića, i na kraju utvrdilo da li prisustvo mutiranog proteina utiče i na ostale proteine.

Rezultati studije su pokazali da se nivo mutirajućeg CASQ2 smanjio brzo nakon rođenja. Takođe je primećeno da je razvijeni oblik proteina bio povezan sa ushodnom regulacijom. Analizirani su važni proteini kod formiranja trijada skeletnih mišića, a takođe su uočeni i povišeni nivoi proteina kod odraslih *knock-in* miševa sa CASQ2-R33Q/R33Q.

Veruje se da je mutacija R33Q dovela do smanjenja CASQ2 putem aktivacije odgovora razvijenog proteina i posledične proteozomalne degradacije.

**Ključne reči:** kalsekvestrin, degradacija povezana sa endoplazmatskim retikulumom, odgovor razvijenog proteina, trijade, haperoni

