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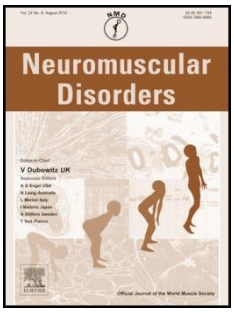

Title: 217th ENMC International Workshop: *RYR1*-related Myopathies, 29-31st January 2016, Naarden, The Netherlands

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217th ENMC International Workshop: *RYR1*-related Myopathies, 29-31st January 2016, Naarden, The Netherlands

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HIGHLIGHTS

The present ENMC workshop report summarizes recent clinical, molecular and genetic advances in the field of *RYR1*-related myopathies, and their current translation into clinical practice for patient benefit.

1. Introduction and overview

17 clinicians and basic scientists from 7 countries, as well as 4 patient representatives, gathered for the 217th ENMC International Workshop on *RYR1*-related Myopathies from 29-31st January 2016 in Naarden, the Netherlands. The skeletal muscle ryanodine receptor *(RYR1)* gene encodes the principal sarcoplasmic reticulum (SR) calcium release channel (RyR1) with a crucial role in excitation-contraction coupling (ECC). Over the last decade, dominant and, more recently, recessive *RYR1* mutations have emerged as the most common cause of non-dystrophic inherited neuromuscular disorders, including various congenital myopathies – Central Core Disease (CCD), Multi-minicore Disease (MmD) and Centronuclear Myopathy (CNM), Congenital Fibre Type Disproportion (CFTD) -, the Malignant Hyperthermia Susceptibility (MHS) trait and exertional rhabdomyolysis (ERM). The present workshop was the 5th ENMC workshop solely dedicated to *RYR1*-related neuromuscular conditions, including previous ENMC workshops on CCD in January 2001 [1], MmD in May 2000 [2] and November 2002 [3], and the various core and other *RYR1*-related myopathies in March 2007 [4] and April 2011 [5], respectively.

Following an introduction by **Alexandra Breukel**, Managing Director of the ENMC, on behalf of all co-organizers **Heinz Jungbluth**, (London, UK) summarized recent developments in the field of *RYR1*-related myopathies (*RYR1*-RMs), and outlined the major themes and objectives of the present workshop. Since the most recent ENMC workshop on *RYR1*-RMs in 2011, the phenotypical spectrum has expanded further, in particular in relation to *RYR1* mutations associated with recessive inheritance, and induced or late myopathic manifestations of dominant *RYR1* mutations previously mainly associated with the MHS trait.

In addition to advances in the understanding of the disturbances of intracellular Ca^{2+} homeostasis and ECC underlying dominant *RYR1* mutations, novel pathogenic mechanisms in particular underlying recessive *RYR1*-RMs are emerging. The first pharmacological compounds are currently being trialled based on these observations, and further clinical trials are likely to follow in the near future. As an important step towards the translation of research findings into patient benefit, the RYR-1 Foundation (www.ryr1.org) was recently established as a support and advocacy group for individuals affected by *RYR1*-related conditions and their families. Following the introduction by the organizers, ***Mike Goldberg*** and ***Jennifer Ryan*** from the RYR-1 Foundation (Pittsburgh, USA) provided a unique patient and family perspective on *RYR1*-RMs, and how their RYR-1 Foundation was established.

2. Clinical spectrum of *RYR1*-related myopathies: genotype-phenotype correlations

Heinz Jungbluth (London, UK) summarized novel, induced and late-onset myopathic phenotypes associated with dominant, mainly MHS-related *RYR1* mutations. Malignant Hyperthermia (MH), a pharmacogenetic predisposition to adverse reactions in response to halogenated anaesthetics and muscle relaxants, and CCD were the first conditions to be attributed to defective RyR1 function. The clinical and mutational overlap between MH and myopathies such as CCD and the King-Denborough syndrome (KDS) [6] has been recognized for some time, but the recently observed consistent association of certain *RYR1* mutations with *(exertional) rhabdomyolysis (ERM)* [7, 8] suggest that ERM may be a more common manifestation of MH-related *RYR1* mutations than actual MH itself. Rhabdomyolysis, sudden catastrophic muscle breakdown in response to various triggers, is commonly attributed to metabolic or other neuromuscular conditions but a substantial proportion of patients remain genetically unresolved despite extensive investigations. Recent

findings suggest that MH-related *RYR1* mutations may account for between 20-30% of cases with ERM, often in individuals with above average athletic abilities but rarely a personal or family history of MH. Common triggers of *RYR1*-related ERM include exercise, increased environmental temperatures, alcohol, drugs - or a combination of the above - but, in contrast to most metabolic causes, not fasting. A recently reported case of a fatal, *RYR1*-related MH reaction in the context of general anaesthesia administered shortly after unaccustomed intense exercise suggests that the latter may also play a role in MH pathogenesis [9]. RYR1-related rhabdomyolysis may also occur in the context of (viral) illness without additional triggers, mimicking viral myositis [7, 10]. Another recently recognized but probably less frequent manifestation of MH-related *RYR1* mutations is *late-onset axial myopathy* [11, 12], characterized by progressive weakness of paravertebral muscles in previously normally strong (or even particularly muscular) individuals; despite a clear association with MH-related *RYR1* mutations, low penetrance and intrafamilial variability of this phenotype suggests the presence of additional (genetic) modifiers, as well as genetic heterogeneity. Lastly, periodic paralysis, previously reported in one isolated case [13], has now been observed in two other patients (unpublished findings) and is likely to be a rare but genuine association in a subset of *RYR1* mutations.

Carsten Bönnemann (Washington, USA) outlined early-onset *RYR1*-related phenotypes. The typical presentation of congenital CCD due to dominant *RYR1* mutations is that of congenital hypotonia and weakness, possibly presenting with hip dislocation at birth. Motor milestones are delayed, but independent ambulation is eventually achieved. Weakness tends to be most prominent in the pelvic girdle and quadriceps, with little involvement of the face and normal extraocular movements. Motor abilities then show some improvement followed by a long period of stability, with possible progression later in adulthood. Severe neonatal presentations were first described in 2003 [14], due to autosomal recessive or dominant *RYR1*

mutations and occasionally associated with neonatal death. One severely affected dominant case had both cores and rods on muscle biopsy. Some severe patients with dominant *RYR1* mutations and central cores may survive the neonatal period but show severe motor involvement, sometimes precluding independent ambulation, and progressive severe early onset scoliosis, but without facial or extraocular involvement. The severely affected patients reported in Romero et al. (2003) in whom ventilation was continued showed motor improvement, eventually acquired some respiratory autonomy and, in one case, independent ambulation after 5 years of age (Ana Ferreiro, personal observation).

Recessive *RYR1* mutations have become increasingly recognized due to the ability to assess the entire *RYR1* coding sequence beyond the known hotspots. Overall there is a tendency for earlier and more severe presentation compared to most patients with dominant mutations [15], however, it has become apparent that recessive *RYR1* mutations are associated with a wide range of clinical phenotypes and pathological histotypes. A given phenotype may be associated with variable histotypes and vice versa; however, some more distinctive associations are emerging. A group of recessive *RYR1* mutations may present with typical central core or with multi-minicore pathology, which may coexist in the same patient either in different muscles at the same time, or in temporal succession. The clinical phenotype ranges from severe neonatal and potentially lethal to later milder presentations, with similar distribution of weakness as seen as in typical dominant CCD, or sometimes including additional hand weakness. The pathological picture of *RYR1*-related centronuclear myopathy (CNM) [16] is characterized by internalized nuclei, sometimes centralized but often multiple per fiber, with unevennness of oxidative stains that can sometimes assume core or multi-minicore like characteristics. Patients characteristically present with external ophthalmoparesis of variable degree, frequently associated with facial weakness. A similar clinical presentation may also be associated with a more purely multi-minicore like histotype.

Patients have also been reported as presenting with a histopathological pattern more resembling CFTD, but still with external ophthalmoparesis [17]. In addition, there are patients presenting with considerable muscle weakness with a histotype more typical of a congenital muscular dystrophy (CMD) or other nonspecific histological manifestations, including striking fiber atrophy but without cores. Despite considerable skeletal muscle weakness in these patients the extraocular muscles are spared, and early progressive scoliosis is prominent. Thus, amongst the recessive *RYR1*-associated phenotypes there may be at least two clinical groups emerging – with and without ophthalmoparesis, with the former being associated with internal/central nucleation or a multi-minicore like pathology, while in the latter group the histotypes may be that of CCD or CMD and other nonspecific histologies. Going forward, an in depth analysis correlating genotypes with phenotypes, histotypes and most importantly pathophysiological consequences (physiotype) will be most helpful in elucidating these complex relationships.

Caroline Sewry (London, UK) presented a summary of the main pathological features of muscle biopsies from patients with *RYR1* mutations. She emphasised the heterogeneity of pathology and the limitations in our understanding of their meaning. In common with other congenital myopathies, necrosis is rarely a feature but the amount of adipose and connective tissue can be variable. In some very young cases these can be extensive, resembling a CMD, but it is unknown if these findings reflect loss of muscle tissue or failure of muscle fibres to form normally. Sampling may also be a factor as differential involvement of muscles is seen with muscle MRI [18, 19]. Internal nuclei and central nuclei are frequently a feature and some cases have been reported to pathologically resemble a CNM. These cases, however, do not often show the pale peripheral halo seen in *MTM1*-related XLMTM on oxidative enzyme stain. The cause and effect of mislocalised nuclei is not known but nuclear positioning can influence gene expression and be influenced by nuclear membrane proteins, microtubules and

the cytoskeleton. Fibre typing in *RYR1*-RMs often shows a uniform pattern or predominance of slow type 1 fibres. Fibre type disproportion without any additional defect may also occur but immunolabelling for myosin isoforms indicates that not all fibres with slow myosin are small and some may co-express fast myosin, indicating a need to re-evaluate fibre type disproportion in terms of myosin isoforms. The cause and effect of type 1 fibre predominance in congenital myopathies is not known, although innervation, the NFAT and calcineurin pathways are known to be involved. As fibre necrosis is rare, regenerating fibres are also not a feature but very small ("pinprick") fibres (less than 5 microns) expressing fetal myosin can be seen, however, those are not specific to *RYR1* myopathies. Cores with disrupted myofibrils and absent mitochondria are the best known feature associated with *RYR1* defects but they can be variable in size or may even be absent. Large cores (single or multiple) running down an appreciable length of fibres are often associated with C-terminal dominantly inherited *RYR1* mutations, whereas small, multiple cores are more often associated with recessively inherited *RYR1* mutations but are not specific. Various proteins accumulate in core areas and in some fibres cores may show intense peripheral labelling of proteins such as desmin. It is then important to distinguish cores from target fibres. *RYR1*-related cases with exertional myalgia and malignant hyperthermia often show only mild, non-specific myopathic pathological features.

Jim Dowling (Toronto, Canada) presented a summary of his published genotype-phenotype correlation study investigating recessive *RYR1*-related myopathies [20]. This study consisted of an analysis of 14 new and 92 published cases. His team (in collaboration with the late Nigel Clarke, Sydney Australia) examined mutation type (missense versus hypomorphic) and location in reference to histopathologic subtype and clinical severity. Overall, approximately 50% of the 106 cases were non-core myopathies, with diagnoses including CNM, CFTD, and other non-specific histopathological findings. Patients with non-core myopathies were more

likely to have at least one hypomorphic mutation (i.e. a mutation that causes a partial loss of gene function), particularly as compared to recessive CCD. Importantly, the presence of at least one hypomorphic allele was significantly associated with increased clinical severity, implying that reduced levels of the RyR1 protein result in more severe disease. In terms of mutation location, while mutations are present throughout the protein, there was an enrichment of missense mutations in the 3 previously described "hotspot" regions affecting the N-terminal, central and C-terminal domains, particularly in patients with core myopathies. Mutations located in hotspot 3 and the triadin binding domain were associated with a more severe clinical phenotype. Lastly, ophthalmoplegia was common across all subtypes, but more frequently seen with hypomorphic mutations. One future direction based on this work will be to perform a more dedicated examination of missense mutations and see whether certain mutations are more highly associated with ophthalmoplegia.

Heinz Jungbluth (London, UK) presented on *RYR1* in the context of other congenital myopathies. Recent epidemiological studies covering different geographical areas have identified *RYR1* mutations as the most common cause of congenital myopathies [21, 22]. Reflective of their overall frequency, different *RYR1* mutations may be running independently in the same family [15], complicating genotype-phenotype correlations and genetic counselling, or may occur in addition to other monogenic conditions [23], emphasizing the importance of considering "double-trouble" in patients with *RYR1*-related myopathies and unexpected additional features. In particular recessive *RYR1*-related myopathies share marked clinico-pathological overlap with *SEPN1*-related myopathies and XLMTM, probably reflecting common defects in redox regulation and triad assembly, respectively. Other congenital myopathies that have to be considered in the differential diagnosis of recessive *RYR1*-related are those due to recessive mutations in the *MYH2* gene [24] if extraocular muscle involvement is present, or in *TTN* [25] or *MYH7* [26, 27] in the

presence of cardiac involvement. Recessive mutations in *STAC3* are a recently identified cause of a KDS-like phenotype with recurrent MH episodes early in life [28].

Nicol Voermans (Nijmegen, The Netherlands) presented the results of a retrospective cohort study investigating the clinical, genetic and histopathological features of all pediatric and adult patients referred to a national centre for both malignant hyperthermia and inherited myopathies over a 5 year period (2008-2012) in whom *RYR1* mutations had been identified [29]. Among 277 patients tested, *RYR1* mutations were detected in 77 non-related patients (detection rate 28%), featuring both congenital myopathies with permanent weakness and 'induced' myopathies ("dynamic presentations") such as MHS and non-anesthesia-related episodes of rhabdomyolysis or hyperCKemia, triggered by various stimuli. Sixty-one different *RYR1* mutations were detected, of which 24 were novel. Some *RYR1* mutations behaved as dominant with regards to the MHS but as recessive with regards to congenital myopathy phenotypes, even within the same family. Age of onset varied from birth to 60 years in dominant and from birth to 10 years in recessive cases. Histopathological features included an equally wide spectrum, ranging from only subtle abnormalities such as increase of internal nuclei or unevenness of oxidative staining, to prominent cores. A series of three subsequent muscle biopsies in one patient showed the development of central cores over time. Some biopsies showed mild inflammatory or mitochondrial changes, obscuring the correct diagnosis. Since publication of the original paper, the Dutch cohort has now extended to 108 non-related patients.

The ERM phenotype (see above) was prominent in the Dutch series and was illustrated in more detail in a separate report [8]. The first patient initially reported as 7.II.2 in Dlamini et al [[7]] and carrying the common European MH mutation Gly2434Arg, has so far suffered six episodes of rhabdomyolysis in response to different triggers, including exercise, stress and viral infections. Her brother, carrying the same mutation, has asymptomatic hyperCKemia

(up to 1200 IU/l) but no rhabdomyolysis history despite a strenuous exercise regime. The second patient, compound heterozygous for *RYR1* mutations p.Val4849Ile and p.Ile2321Val, experienced recurrent rhabdomyolysis episodes after cycling (CK up to 28000 IU/l). His father, a carrier of the *RYR1* p.Val4849Ile mutation was asymptomatic, but his uncle had suffered from exercise-induced myalgia for years; he also carried the p.Val4849Ile mutation and had a mildly myopathic muscle biopsy, suggesting large intrafamilial variability. Late onset axial myopathy (see above) [11, 12] was also prominent in the Dutch cohort, with four patients presenting between the third and sixth decade with predominant axial muscle involvement, comprising variable degrees of lumbar hyperlordosis, scapular winging and/or camptocormia; in one case, scapular winging was markedly asymmetric, mimicking facioscapulohumeral dystrophy (FSHD).

Phil Hopkins (Leeds, UK) presented genotype-phenotype correlations in hyperthermic manifestations of *RYR1*-related myopathies. Hyperthermia is defined as an increase in body temperature in the presence of a normal central thermoregulatory set-point, and can result from abnormal heat gain, a failure of heat dissipation, or a combination of both. Life-threatening hyperthermia is called heat stroke but clinical diagnosis of heat stroke requires an assessment of cerebral function. During general anaesthesia, it is not possible to clinically assess cerebral function and so the development of a progressive life-threatening hyperthermic reaction under anaesthesia requires an alternative name. This is why the classical hyperthermic manifestation of *RYR1* myopathy, which occurs during general anaesthesia, is known as malignant hyperthermia (MH) and not heat stroke. Genotype-phenotype relationships in MH-susceptible individuals can be explored using data obtained from the diagnostic *in vitro* muscle contracture tests. Results from such analyses show that the degree of *in vitro* muscle response depends on the specific *RYR1* mutation carried by the patient. There is also evidence that gene dose affects the phenotype: For example, the *RYR1*

p.R3772Q mutation is associated with MH in the heterozygous state but in the homozygous state there is also a clinical myopathy. In a cohort of 62 individuals with a history of exertional heat illness and abnormal thermoregulation as determined by a standardised heat tolerance test, the Leeds team have identified nine individuals with rare and potentially pathogenic *RYR1* variants. Work is ongoing to determine the functional significance of these variants. Recent publications have re-awakened interest in the concept of a human stress syndrome with *RYR1* aetiology. It must be emphasised that, by definition, these cannot be termed "awake" malignant hyperthermia reactions. The reported cases had features of muscle hypertrophy and the reactions were notable for early muscle rigidity often associated with a febrile or viral illness, observations that parallel the porcine stress syndrome (PSS). In contrast, in the typical human MH response under anaesthesia, muscle rigidity is a relatively late manifestation of the hypermetabolic and hyperthermic syndrome.

3. *RYR1* sequence variation and human disease

Phil Hopkins (Leeds, UK) presented the analysis of *RYR1* variants in the UK MH cohort. The latest European Malignant Hyperthermia Group (EMHG) diagnostic guidelines have introduced the option of primary DNA screening for the index case, a possibility largely arisen through the cost-effectiveness of next-generation sequencing (NGS) technology. Even before the introduction of NGS, only a minority of the more than 200 MH-associated *RYR1* variants had been shown to produce a functionally relevant defect. Such functional studies are vital before variants can be used diagnostically because *RYR1* is a genetically diverse gene in which rare, potentially pathogenic variants are found in up to 6% of control populations. The Leeds team have used NGS to sequence the entire *RYR1* coding region in a cohort of 126 unrelated UK MH families who had undergone a variable degree of previous genetic analysis.

RYR1 variants were found in 72 samples but only on 20 of these were the variants known to be pathogenic or likely to be pathogenic. Sixteen variants were known polymorphisms or unlikely to be pathogenic, while 50% of the *RYR1* variants found were of unknown significance.

Sheila Riazi (Toronto, Canada) presented on malignant hyperthermia (MH), exertional heat illness (EHI) and *RYR1*-related myopathies: distinct entities or a continuum? MH, a potentially fatal pharmacogenetic disease, is classically triggered by volatile anaesthetics. During an MH crisis the dysregulated rise of myoplasmic Ca^{2+} leads to hypermetabolism, through yet undefined pathways. Despite the reports showing a possible connection between EHI and MH, distinction between these entities is challenging, due to variations in clinical presentation, inconclusiveness of genetic testing, and the fact that caffeine halothane contracture test (CHCT) is not designed to diagnose EHI. However, it is vital to know if MH patients should be warned against exercise in heat, or if EHI patients should avoid volatile anaesthetics. In this proof of concept study, Dr Riazi and colleagues compared hypermetabolic details in anaesthetic induced and non-anaesthetic induced MH patients with controls, on the cellular level by exploring Ca2+ movements in muscle cells and through metabolomics, and on the clinical level by *in vivo* assessment of muscle metabolism using ^{31}P-magnetic resonance spectroscopy. Applying such a multipronged approach, they showed that measurement of Ca2+ concentration and fluxes in conjunction with clinical information can be used to classify these patients more precisely. More specifically, metabolomics study showed impairments of both carbohydrate and fatty acid metabolism pathways in EHI but even more so in anaesthetic-induced MH patients. The markers of oxidative stress were significantly higher in MH and EHI, compared to controls. Finally, *in vivo* techniques of muscle tissue metabolism were indicative of impaired oxidative phosphorylation pathways both in MH and EHI patients. These preliminary results have established the feasibility and

accuracy of the applied approach. This project is the first step towards finding similarities and common target in these heterogeneous disorders.

Susan Treves (Basel, Switzerland) presented recently reported findings concerning novel pathogenic mechanisms in recessive *RYR1*-RMs, obtained through a study to gain mechanistic insight into the causes of the reduced RyR1 content commonly observed in recessive *RYR1*-RMs. Muscle biopsies from patients with recessive *RYR1* mutations were found to exhibit decreased expression of muscle specific microRNAs, increased DNA methylation of one of the CpG islands within the *RYR1* gene, and increased expression of class II histone deacetylases; the latter event was accompanied by down-regulation of histone acetylation and by increased nuclear co-localization of HDAC-4 and mef-2. Transgenic mouse muscle fibres over-expressing HDAC-4/HDAC-5 for 1 week exhibited decreased expression of *RYR1* and of muscle specific miRNAs, while acute knock-down of *RYR1* in mouse muscle fibres by siRNA caused up-regulation of HDAC-4/HDAC-5. These results indicate that a pathophysiological pathway caused by epigenetic changes is activated in the muscles of some patients with recessive *RYR1*-RMs. Future investigation will be aimed at unravelling the mechanism by which *RYR1* mutations activate this epigenetic pathological loop and verifying whether DNA methyltransferases and class II HDACs could be targeted pharmacologically to improve the muscle function in this group of patients.

4. The basic science of *RYR1*-related myopathies

Andy Marks (New York, USA) presented recent findings from his team concerning the crystal structure of mammalian ryanodine receptors and Rycals as a treatment option for *RYR1*-RMs. RyR channels are required for release of calcium from intracellular stores, a process essential for many cellular functions including excitation-contraction (EC) coupling

in skeletal and cardiac muscle, and hormone and neurotransmitter release. RyRs are amongst the largest ion channels, comprised of the four identical ~565 kDa channel-forming protomers, as well as regulatory subunits, enzymes and their respective targeting/anchoring proteins, in a macromolecular complex that exceeds three million Daltons. Andy Marks' team have obtained high-resolution cryo-electron microscopy (Cryo-EM) reconstructions from highly purified rabbit skeletal muscle RyR1 in the open and closed states. These data reveal that RyRs are members of the six transmembrane family of ion channels and show a mechanism for channel gating that couples a change in the conformation of the Ca^{2+} binding site directly to opening of the channel pore, suggesting that Ca^{2+} binding facilitates mechanical coupling of conformational changes in the cytosolic region to opening of the channel gate. The channel-specific ligand ryanodine shows the channel to be locked in an open state consistent with the know effects of ryanodine on the single channel properties of RyR1. Their team have mapped >180 disease causing mutations on the structure proving insight regarding disease mechanisms for arrhythmias and muscular dystrophies. Unpublished clinical data on patients and RyR1 single channel studies have been conducted to better understand how mutations in the skeletal muscle RyR1 cause *RYR1*-RMs. This information may also yield insights as to which disease causing mutations are likely to respond to treatment with Rycals, a novel class of RyR stabilizing drugs, for guiding future clinical trials. Ca^{2+} leak through dysfunctional RyR1 may affect gene expression, protease activity, and redox homeostasis and has been linked to several myopathic conditions including Duchenne muscular dystrophy, sarcoglycanopathies, and sarcopenia. In these conditions secondary, post-translational modification of RyR1 causes pathologic Ca^{2+} leak leading to myopathy. Stabilizing RyR1s with Rycals reduces Ca^{2+} leak and improves muscle function in mice with these myopathies.

The presentation from **Robert Dirksen** (Rochester, USA) started with a historical recount of

how analyses of *RYR1* disease mutations have provided clues into important functional regions of the RyR1 Ca^{2+} release channel. For example, the discovery of the *RYR1* I4898T mutation in a large Mexican family with CCD [30] quickly led to the identification of the selectivity filter of the RyR1 channel [31]. The I4898T mutation was subsequently shown to reduce RyR1 Ca^{2+} release by altering Ca^{2+} permeation ("EC uncoupling") of the channel [32]. Similarly, functional studies of MH and CCD mutations in the N-terminal and central regions of RyR1 [33, 34] led to the idea that some disease mutations promote RyR1 Ca^{2+} sensitivity to opening ("Ca^{2+} leak") by disrupting critical inter-domain interactions important for stabilizing the channel closed state [35, 36]. The recent use of cryo-electron microscopy to obtain high resolution structures of RyR1 by three groups in early 2015 was a major breakthrough for the field as these structures provide unprecedented insight into the mechanisms by which *RYR1* disease mutations alter RyR1 channel function [37-39]. Bob Dirksen presented unpublished collaborative biochemical and functional studies for specific MH- and CCD-related *RYR1* mutations located in regions of the high resolution RyR1 structure predicted to either promote channel activity by stabilizing the channel open state (Ca^{2+} leak) or reduce Ca^{2+} release by stabilizing the channel closed state (EC uncoupling). It is expected that the continued refinement of high resolution structures of the RyR1 open and closed states will provide additional important new insights into the pathophysiological mechanisms of *RYR1*-related disorders.

Francesco Zorzato (Ferrara, Italy) presented results from a recent study describing altered calcium homeostasis in arterial smooth muscle cells (SMCs) in the MH Y522S mouse model (unpublished findings). Since RyR1 is expressed not only in skeletal muscle, *RYR1* mutations may lead to alterations of Ca2+ homeostasis also in other tissues expressing this intracellular calcium release channel, even if at lower levels. Prompted by the clinical observation of an increased bleeding tendency in some patients with MH-related *RYR1* mutations (see below),

biochemical characterization and immunofluorescence analysis of RyR1 in arterial smooth muscle cells were performed in the Y522S mouse model. Aorta and tail artery were found to express the RyR1 transcript, although, as expected, to a much lower extent than skeletal muscle. Tail artery smooth muscle cells isolated from the RYR1 Y522S knock-in mouse exhibited increased frequency of localized calcium release events (sparks) and smaller intracellular Ca2+ stores compared to WT littermates, although the resting calcium concentration was not affected. Calcium sparks frequency was also found to be increased and associated with a more negative membrane potential in smooth muscle cells from RyRY522S mice compared to SMCs isolated from WT littermates. These findings demonstrate that MH-associated *RYR1* mutations alter vascular smooth muscle cell function by affecting calcium homeostasis.

5. Animal models and therapy development for *RYR1*-related myopathies

Susan Hamilton (Houston, Texas) presented her work on therapy developments using mouse models of *RYR1* myopathies. Mutations affecting the type I ryanodine receptor (RyR1) are associated with human muscle diseases including MH, MH with cores, CCD, MmD, and others. Some CCD mutations increase SR Ca^{2+} leak, but others decrease Ca^{2+} permeation through RyR1, raising the question of how mutations that lead to opposing functional effects on RyR1 can result in related disease phenotypes. Dr. Hamilton described the use of two mouse models of *RYR1*-RMs (Y522S and I4898T) to assess mechanisms of disease and develop new interventions. In particular, she discussed efficacy of AICAR [40], N-acetylcysteine [41], and low dose rapamycin [42] for improving muscle function in these modes models. She also discussed recent findings with the I4898T mice (a RyR1 pore blocking mutation) and the need for very different approaches to improving muscle function

in these mice than those used with the Y522S mice with Ca^{2+} leaky mutations. She discussed both progress toward developing drugs that improve muscle function in mice with RyR1 pore blocking mutations and the potential of CRISPR technology to eliminate or edit the mutated allele in these mouse models of dominantly inherited *RYR1*-RMs. The work with the mouse models suggest that, at least in mice, the *RYR1*-RMs are progressive and that different mutations reduce muscle function by different underlying mechanisms. These findings suggest the need for mutation-specific interventions.

Ana Ferreiro (Paris, France) summarized and discussed the role of oxidative stress as a pathophysiological mechanism in *RYR1*-RMs and its relevance as a therapeutic target. She stressed the clinical and histological overlap between *RYR1*-RMs and myopathies associated with defects in *SEPN1*, encoding the putative antioxidant protein selenoprotein N, suggesting that common pathophysiological pathways, including defects in redox homeostasis, are involved in both conditions. The ryanodine receptors are exquisitely sensitive redox sensors, due to their exceptionally high content in cystein residues (404 per tetramere), which represent a large number of free thiols whose oxidation or nitrosylation influences channel function. Moreover, several sets of cystein thiols have differential susceptibility to redox modifications by PO2, nitric oxide, the ratio between reduced and oxidized glutathion (GSH/GSSG) or S-nitrosoglutathione (GSNO), which provides fine functional modulation of RyRs in response to different redox-related changes in the extracellular and intracellular environment. Excessive and continuous accumulation of reactive oxygen and nitrogen species can alter this physiological regulation, leading to RyR hypernitrosylation and, if unchecked by the antioxidant defence system, ultimately to oxidative muscle injury. Such mechanisms play an important role in physiological conditions such as fatigue after exercise [43] but also in *RYR1*-related skeletal muscle dysfunction. Thus, it has been demonstrated that oxidative stress (reduced GSH/GSSG) and redox remodeling of RyR1 (increased RyR S-

glutathionylation and nitrosylation) cause calcium leak in vitro and mitochondrial injury in RYR1-MH mutant mice [41]. In addition, collaborative research between the groups of Ana Ferreiro and Jim Dowling established that oxidative stress is involved also in *RYR1*-RMs [44]. The total level of reactive oxygen species, mainly of mitochondrial origin, and of carbonylated/oxidated proteins is increased both in myotubes from *RYR1*-mutated patients and in the *ryr* zebrafish. Treatment with the antioxidant N-acetylcysteine (NAC) reduced oxidative stress markers and improved survival in oxidant conditions in *RYR1*-mutated myotubes, ameliorated aspects of the *ryr* motor phenotype and reduced the ultrastructural abnormalities (ER dilatation, minicores) in muscles from the zebrafish model [44]. These studies established that oxidative/nitrosative stress is a relevant and targetable pathomechanism in *RYR1*-RMs, and that NAC is an effective *ex vivo* and *in vivo* treatment in relevant animal models, thereby laying the basis for current clinical trials and for future therapeutic approaches. Ana Ferreiro discussed the need for additional studies to establish the correlation between the type of *RYR1* mutation and its redox-related functional consequences, in order to identify biomarkers and outcomes useful for future personalized therapeutic approaches.

Jim Dowling (Toronto, Canada) discussed small animal models of *RYR1*-related myopathies and their application for drug discovery. He briefly presented the relatively relaxed (*ryr*) zebrafish model, which has a insertion/frameshift mutation in *ryr1b*, and was used for discovering N-acetylcysteine as a potential therapy for patients with *RYR1*-RMs [44]. He then introduced the *unc68* nematode model, a C. elegans mutant strain with a loss of function mutation in *ryr*. He described a sensitized drug screen using this model, and presented the preliminary results of a large-scale screen that he (with collaborator Peter Roy, University of Toronto) recently performed. He concluded by describing several new mutants, both in C. elegans and in zebrafish, that his laboratory is in the process of generating. These new

mutants will more accurately model human *RYR1*-related myopathies by having engineered point mutations that have been described in patients with different subtypes of disease.

Julien Fauré from the team of Isabelle Marty (Grenoble, France) presented genomic medicine approaches to *RYR1* therapeutics. *RYR1*-related pathologies are heterogeneous in their presentation and genetic bases. Although promising molecular tools are being developed for *in vivo* DNA editing, several barriers have to be removed to reach clinical trial readiness for these techniques. Conversely, RNA-based therapies are already tested in phase III clinical trials and could provide a promising therapeutic approach. The team from Grenoble has studied a pathological situation in which an affected child harbors two recessive *RYR1* mutations, resulting in a massive RyR1 reduction, and has developed an exon-skipping strategy with a modified U7-AON to block the effect of the paternally inherited mutation (which induces the inclusion of a new in frame exon in the *RYR1* mRNA, resulting in the insertion of additional amino acids and destabilization of the protein) in affected primary muscle cells. Expression of the U7-AON in primary patient muscle culture resulted in reduced inclusion of the additional exon, an increase in RyR1 protein expression, and restoration of normal calcium releases. This study is the first demonstration of the potential of exon skipping for a recessive *RYR1*-RM. Further steps required to reach clinical trial readiness were presented and discussed.

Francesco Muntoni (London, UK) reported data on targeting dominant *RYR1* mutations using gapmer oligonucleotides, specifically designed to target alleles carrying the mutations and induce RNase-H mediated RNA cleavage. The strategy followed in collaboration with Dr Haiyan Zhou in the London laboratory consists in targeting common single nucleotide polymorphisms (SNPs) in linkage disequilibrium with the mutant allele. If the targeting of the SNP is successful, this should theoretically leave the wild type allele intact, providing a potentially interesting approach for dominant *RYR1*-RMs. Proof of concept has been obtained

so far in an immortalized myoblast cell line derived from a patient carrying a dominant mutation in *RYR1* exon 91, with RNA sequencing clearly indicating the possibility to achieve allele specific silencing using the gapmers.

6. Non-skeletal muscle presentations of *RYR1*-related myopathies

In addition to striated skeletal muscle, RyR1 receptors are expressed in a range of other tissues but non-neuromuscular manifestations associated with mutations in *RYR1* have so far not been reported. **Heinz Jungbluth** (London, UK) and **Nicol Voermans** (Nijmegen, Holland) presented non-neuromuscular manifestations (including bleeding abnormalities, smooth muscle, CNS and cardiac involvement) seen in their cohorts of *RYR1* mutated patients in the United Kingdom and the Netherlands.

Heinz Jungbluth presented evidence for a novel, *RYR1*-associated bleeding abnormality. Assessment of bleeding abnormalities through a standardized bleeding questionnaire (MCMDM-1VWD) demonstrated an increased bleeding tendency in patients carrying *RYR1* gain-of-function mutations compared to normal controls (unpublished findings). The observed bleeding phenotype was more pronounced in *RYR1*-mutated females, characterized by severe menorrhagia and post-partum hemorrhage, and often associated with other signs of smooth muscle involvement affecting bowel and bladder. Corresponding to findings in *RYR1*-mutated humans, bleeding times in the MHS $RYR1_{Y522S}$ knock in mice exhibited 3 times longer bleeding times compared to their wild type littermates, probably reflecting impaired SMC involvement in these animals (collaborative work with Susan Treves and Francesco Zorzato, see above). Most importantly, the bleeding phenotype could be complete reversed by administration of the RyR1 antagonist Dantrolene, suggesting Dantrolene as a therapeutic option for *RYR1*-related and potentially other bleeding disorders.

Other recently observed non-neuromuscular manifestations of *RYR1* mutations include severe CNS involvement in an adolescent suffering a fatal MH episode [9]; the pattern of prominent cerebellar involvement seen in this patient showed striking similarities with neuropathological changes described in deceased heat stroke victims [45]. An intriguing potential link between *RYR1*-related ERM and the Neuroleptic Malignant Syndrome (NMS) is indicated by the implication of certain psychopharmacological drugs such as Olanzapine as triggering agents in individuals with ERM and confirmed pathogenic *RYR1* mutations [7].

Nicol Voermans presented evidence for cardiac involvement in the Dutch cohort of patients with *RYR1*-RMs. In four unrelated patients within this cohort, the family history revealed sudden death at a relatively early age in one or more family members. Post-mortem studies were not performed in any of the deceased individuals, and the sudden death was considered to be of cardiac origin. There were no symptoms suggestive of a non-anaesthetic MH reaction. These patients included the brother of a patient with a late-onset axial myopathy due to the *RYR1* p.Val4849Ile mutation who died suddenly at the end of running a marathon. Other cardiac features in the same series included dilated cardiomyopathy presumed to result from a viral infection, bicuspid aortic valve, and sinus bradycardia. The cause of these observations is unclear, as the main cardiac isoform in the heart is *RYR2* and cardiac *RYR1* expression is limited. Smooth muscle cell involvement in arteries and arterioles (see above) could be a possible explanation. A possible cardiac phenotype associated with *RYR1*-RMs ought to be investigated further.

7. Clinical trials in *RYR1*-related myopathies

Francesco Muntoni (London, UK) presented data on the challenges to clinical trials for rare neuromuscular diseases. He remarked that so far only single centre limited data exist on the

natural history of *RYR1*-RMs, and that outcome measures for these conditions have not been widely characterised. There are no large international registries, but a collection of smaller national ones; and there are no biomarkers that have been validated and that could be used as surrogate markers to monitor disease progression and response to therapy. All these are bottlenecks to trial readiness. In other neuromuscular disorders, for example several forms of muscular dystrophies and spinal muscular atrophy, significant resources have been concentrated in registries and longitudinal multicentre collection of core clinical data from the relevant patient population; on studies of biomarkers; and on testing of outcome measures in a multicentre setting. These are very helpful steps in facilitating at a later stage the involvement of industrial partners, as the lack of the above deter from considering this an area worth exploring for therapeutic developments

Katy Meilleur (Washington, USA) reported on the currently ongoing clinical trial of N-acetylcysteine (NAC) in *RYR1*-RMs in the USA. In preclinical models of *RYR1*-RMs, including mice, zebrafish and patient myotubes, treatment with NAC, an FDA approved drug for various indications, has shown decreased oxidative stress plus increased muscle force [41] and increased swim endurance [44]. Additionally, numerous human trials of NAC in healthy and other diseased populations have shown decreased oxidative stress [46, 47] and decreased time to fatigue [48]. Given the disabling nature of congenital myopathy symptoms, the limited side effect profile of orally administered NAC, and the lack of any FDA approved treatment for these conditions, the benefit of performing a clinical trial in individuals with *RYR1*-RMs outweighs the risk. However, lack of validated clinical outcome measures and biomarkers in this population pose a barrier to clinical trial readiness. Thus, a two-phase study including an initial natural history/outcome measure validation phase followed by a randomized, double-blind, placebo controlled phase is being conducted. Subjects are seen at baseline, 6 months, and 12 months. All outcome measures are performed off-drug at the first

two visits. At the end of the second visit, patients start drug or placebo for the following 6 months and return at 12 months to perform all outcome measures on intervention. Primary outcome measures, chosen based on work in preclinical models, include plasma glutathione (GSH:GSSG) by mass spectrometry for oxidative stress (Specific Aim 1) and 6 Minute Walk Test (6MWT) for endurance (Specific Aim 2). Additionally, several secondary and exploratory outcomes are performed for each specific aim with the goal of validation against the primary outcome measures, which serve as gold standards. So far, 32 participants have been enrolled in the study, including 17 adults, and 15 children. The majority of participants (n=23, 72%) have CCD (n=21, 66%) or MmD (n=2, 6%), or other *RYR1*-RM phenotypes (n=9, 28%). Results based on analysis of the first 32 participants indicate that regarding Aim 1, baseline values for GSH:GSSG are significantly decreased (13.2+5.8) compared to reported healthy controls (21.3+10.3; $p<0.05$), suggesting this endogenous antioxidant is depleted in *RYR1*-RM due to mitochondrial oxidative stress, as seen in the 3 preclinical models. Adults and children in the current study did not show a significant difference in GSH:GSSG. Regarding Aim 2, participants analysed so far had a total mean distance on the 6MWT of 479m (±132m), with adults averaging 481m (±127m) and children averaging 476m (±142). 8 subjects (5 adults and 3 children) reached > 95% predicted distance. Several secondary and exploratory measures for oxidative stress and endurance showed preliminary correlations with their respective gold standard measure. Results showing significant depletion of GSH:GSSG are encouraging as they confirm findings in preclinical models and allow for the potential of NAC to replenish these levels. For more mildly affected participants, the 6MWT reaches a ceiling effect. If necessary, the ceiling effect of the 6MWT may be overcome by analyzing inter-individual differences, as planned in the original study design, and/or by performing a subgroup analysis of more affected individuals who achieve less than 80-90% predicted distance.

Ana Ferreiro (Paris, France) presented the clinical trial using NAC to treat *SEPN1*-related myopathies (*SEPN1*-RM) (SELNAC) which is currently in progress in France (ClinicalTrials.gov Identifier:NCT02505087). Based on the identification by her group of oxidative stress as a relevant pathophysiological mechanism, and of cystein-donors and particularly NAC as an effective treatment *ex vivo* in *SEPN1*-RM, a preclinical study was conducted using the *SEPN1* KO mouse model. This study demonstrated improvement of several muscle function parameters upon treatment with moderate NAC dosages, and led to the identification of systemic biochemical changes that represent potentially useful biomarkers (Dill et al., in preparation). These parameters will be used to monitor and quantify response to treatment in the SELNAC trial, which is a phase II-III, randomized, double blind, placebo-controlled, cross-over study on 24 French adult patients. Detailed motor and respiratory function parameters will also be quantified before and after treatment. The possibility of extension of this or similar studies to the paediatric *SEPN1*-RM population were discussed. Furthermore, Dr. Ferreiro presented data about clinical trial readiness in the French *RYR1*-RM population, including over 100 patients.

8. Conclusions, outcomes and deliverables from the workshop

Although a number of relatively large retrospective single centre series have already been published, large prospective multicentre data concerning the natural history of *RYR1*-RM are still lacking and pose a considerable obstacle for trial readiness. Moreover, reflecting the immense clinical heterogeneity of *RYR1*-RM, there is no single assessment tool with the ability to robustly collect information from profoundly weak to ambulant patients, resulting in the need to adapt already existing tools and to define suitable functional outcome measures for these widely heterogeneous conditions. There is also a need to determine additional

biomarkers for *RYR1*-RM, both concerning monitoring of general disease progression (for example through serial muscle MRI) but also with regards to pathways targeted by specific drugs (for example in plasma or on muscle biopsies). One deliverable of the workshop was therefore to establish a consortium specifically dedicated to a multicentre natural history study and definition of biomarkers and outcome measures. A first objective of this consortium will be to examine more precisely potential genotype correlations for example with ophthalmoparesis, a study designed to both unify existing patient cohorts and to serve as a springboard for future genotype-phenotype correlation studies.

In addition to pharmacological compounds already being trialled such as acetylcysteine, the need to consider clinical trials for compounds currently being investigated at the pre-clinical stages (for example, Rycals and AICAR [40]) or previously tested in small pilot studies or single cases only (for example, Salbutamol [49] and Dantrolene [7, 8]) was discussed.

Another recurrent theme throughout the workshop was the considerable challenge of correctly assigning pathogenicity to the large numbers of *RYR1* variants currently being identified due to recent advances in genetic diagnostics, and the lack of a central repository for the collection of reliable genotype-phenotype data. To assess pathogenicity more reliably, collaborations between clinicians and basic scientists were established at the workshop, to pursue a more in depth study of selected *RYR1* variants through combined analysis of clinico-pathological, functional and crystallographic data. Already existing genotype-phenotype databases were discussed and further enquiries will be made as to the most suitable currently existing databases for adaptation as a *RYR1* genotype-phenotype repository. Another deliverable of the workshop in relation to this topic is to partner with an existing NIH-led effort (Dr Les Biesecker, Bethesda, USA) to develop algorithms for assessing *RYR1* variants.

Another important question identified at the workshop is the uncertainty regarding the malignant hyperthermia (MH) risk potentially associated with the large number of novel, in

particular recessive *RYR1* mutations currently being identified; the anaesthetists and clinicians present at the workshop agreed to produce a joint position paper to provide practical guidance on this complex issue. In addition to the potentially increased MH risk, an increased bleeding tendency, sudden unexplained death and a substantial disease burden through pain and fatigue were identified as potentially important disease associations that will warrant more systematic study through a validated questionnaire approach.

Lastly, patient representatives present at the workshop emphasized the importance of wider awareness of standards of diagnostics and care concerning *RYR1*-RM and other congenital myopathies, and this will be achieved through wider dissemination of two recent international consortia papers [50, 51] covering these topics. There was also discussion of enhancing awareness of and participation of patient registries, and particularly the registry established by the RYR1-Foundation, as well as working toward development (in part through the registry) of a global natural history study.

Abbreviations:

CCD, Central Core Disease; CNM, Centronuclear Myopathy; CFTD, Congenital Fibre Type Disproportion; ECC, Excitation-Contraction Coupling; EMHG, European Malignant Hyperthermia Group; ERM, exertional rhabdomyolysis; KDS, King-Denborough syndrome; MH, Malignant Hyperthermia; MHS, Malignant Hyperthermia Susceptibility; MmD, Multi-minicore Disease; NMS, Neuroleptic Malignant Syndrome; PSS, porcine stress syndrome; *RYR1*, skeletal muscle ryanodine receptor gene; RyR1, skeletal muscle ryanodine receptor;

RYR1-RM, *RYR1*-related myopathies; SMCs, smooth muscle cells; SR, sarcoplasmic reticulum

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