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REVIEW ARTICLE

ANO5-related muscle diseases: From clinics and genetics to pathology and research strategies

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Abstract Anoctamin-5 (ANO5) is a multi-pass membrane protein localized to the sarcolemma and the sarcoplasmic reticulum. Mutations were linked to rare autosomal recessive muscle diseases. Here, we summarize the clinical spectrum, imaging data and molecular research findings as well as results of animal modeling, which significantly moved forward the understanding of mechanisms underlying *ANO5*-related muscle diseases. Given that precise histological information on inflammatory processes taking place in patient-derived muscle are still lacking, an (immuno)histological study on biopsies derived from six *ANO5*-patients was performed showing focal accumulation of necrotic fibers, mild fiber-size variances and myophagocytosis. In addition, MRI data of four *ANO5*-patients (including a 10-year follow-up in one patient) are presented and discussed in the context of previously published MRI-

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findings. Hence, data presented in this article combining a review of the literature with own myopathological findings address scientific trends and open questions on ANO5-related muscle diseases, which would be of significant interest for a wide neuromuscular diseases community. To conclude, a clear genotype—phenotype correlation does not exist, and *ANO5*-related muscle disorders might represent the next entity of a clinical continuum with varying degree of muscle cell pathologies. In addition, results of pre-clinical studies allowed the definition of suitable cell and animal models characterized by certain histological and functional pathologies resembling the human phenotype. These models might serve as suitable systems for testing of interventional concepts in future.

Introduction

Anoctamins, are also termed TMEM16 (transmembrane proteins with 16 domains), represent a group of at least 10 members of intracellular-calcium-activated proteins with diverging functions. All Anoctamins present with eight transmembrane domains and a DUF590 domain of unknown function.[1] The ten members of the group are termed from ANO1/TMEM16A to ANO10/TMEM16K and display a "clear-cut expression pattern" throughout human tissues.[2] The Anoctamins are involved in a variety of physiological processes including ion transport, phospholipid scrambling as well as regulation of the function of other ion channels. However, the exact function of Anoctamins is still under research with ANO1 and ANO2 as the best studied members thus far: both encode cytosolic Ca^{2+}-activating chloride channels and regulate epithelial transport, smooth muscle contraction, pain sensation and cell proliferation.[3] Other members of this protein family such as ANO6 are known to mediate Ca^{2+}-dependent exposure of phospholipids on the extracellular surface which are usually expressed on the inner leaflet,[2] a physiological process termed as phospholipid scrambling and thus suggesting that ANO6 functions as a scramblase required for blood coagulation.[4] Anoctamin-5 (ANO5), a further member of this protein family, is encoded by a 22 exons-spanning gene and known to be expressed in bones, testes, thyroid, skeletal and cardiac muscles (Fig. 1) functioning as a sarcoplasmic/endoplasmic reticulum (ER)-associated putative intracellular Ca^{2+}-activated chloride channel.[3] Interestingly, ANO5 has been shown to regulate cell migration and invasion in thyroid cancer[5] as well as cell proliferation and migration in pancreatic cancer.[6] Further information about the function of ANO5 in (skeletal) muscle are provided below.

Clinical and genetic findings

Review of the literature

Phenotypes caused by ANO5 mutations

Dominant mutations in *ANO5* are linked to the manifestation of a rare skeletal disorder termed gnathodiaphyseal dysplasia (MIM: 166260). This rare skeletal syndrome is characterized by bone fragility, cement-osseus lesions of the maxilla and mandibula, and diaphyseal sclerosis of tubular bones.[1]

Here, we aim to focus on the different neuromuscular phenotypes/symptoms as well as further co-morbidities such as cardiac involvement caused by recessive mutations in the *ANO5* gene. Toward this aim, we summarized clinical

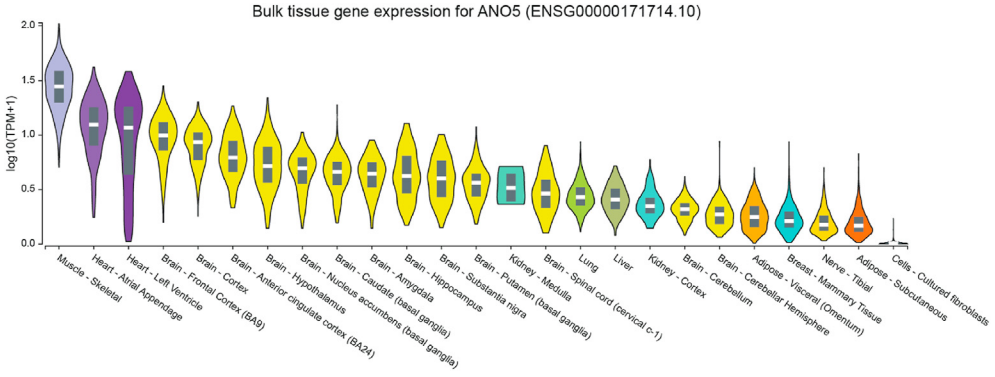

Figure 1 GTEx-based *in silico* analysis of tissue expression of *ANO5*. Expressed are log10-ratios of transcripts per million (TPM) in the respective tissues/nervous areas as violin plots.

Table 1 Summary of clinical findings in a cohort of 37 *ANO5*-patients (modified presentation of clinical data published by Silva and co-workers[7]).

Clinical characteristics	Frequency
Male	27/37
Female	10/37
Adult onset (>21)	31/37
HyperCKemia	37/37
Atrophy	24/37
Proximal weaknessrowhead	24/37
Mild proximal weakness (MRC 4)	22/37
Moderate to severe proximal weakness (MRC < 4)	2/37
Distal weaknessrowhead	10/37
mild distal weakness (MRC 4)	8/37
Moderate to severe distal weakness (MRC < 4)	2/37
Functional statusrowhead	
Ambulant	31/37
Assisted walking	5/37
Wheelchair bound	1/37
Asymmetry	13/37
Dysphagia	2/37

data of 37 patients with confirmed mutations in *ANO5* published by Silva and co-workers in Table 1.[7]

Limb girdle muscular dystrophy subtype 12 – LGMDR12

In 2007, Hu and colleagues linked a novel autosomal recessive form of limb-girdle muscular dystrophy with quadriceps atrophy to chromosome 11p13-p12[8] and in 2010, recessive mutations in *ANO5* have been linked to the phenotypic manifestation of proximal limb-girdle muscular dystrophy (LGMDR12 formally LGMD2L; Muscular dystrophy, limb-girdle, autosomal recessive 12; MIM: 611307) in three French Canadian families as well as to a distal non-dysferlin Miyoshi myopathy (MMD3) in Dutch and Finnish families.[1] The MMD3 phenotype is further described below.

LGMDR12 has clinically been described in multiple publications and is characterized by asymmetric atrophy and weakness of quadriceps and biceps muscles. The mean age of onset is 35 years, but the range of phenotypical manifestation can be between childhood and the sixth decade of life,[1,9–12] complicating the suspicion of an *ANO5*-caused LGMD during clinical examination. Commonly the muscular involvement is preceded by a time of hyperCKemia with or without myalgia which can range from month up to decades.[10] Most common manifestations of muscle weakness can be observed on iliopsoas or hip flexors muscles followed by quadriceps or knee extensors muscles.[1,8–10] In a cohort of 13 LGMDR12 patients, seven presented with reduced muscle strength in elbow flexion, which is usually accompanied by reduced strength in shoulder abduction. However, of these seven patients, only four presented with reduced shoulder abduction strength.[9] Notably, such a preserved function in this relationship of muscles has not been described in LGMD patients before. In accordance with an LGMD-phenotype, distal weakness is only very rarely observed in LGMDR12 patients with asymmetric atrophy and hypertrophy of the calf muscles[1,8–10,13]; distal weakness in both, upper and lower limbs have not been described thus far. Clinical data of 20 patients with confirmed genetically confirmed LGMDR12 published by Hicks and co-workers[13] were summarized in Table 2.

In brief:

- LGMDR12 is characterized by asymmetric atrophy and weakness of quadriceps and biceps muscles.
- The mean age of onset is 35 years.
- Muscular involvement is commonly preceded by a time of hyperCKemia.
- Most common manifestations of muscle weakness can be observed on iliopsoas or hip flexors muscles followed by quadriceps or knee extensors muscles.
- Distal weakness is only very rarely observed in LGMDR12 patients.

Miyoshi myopathy distal subtype 3 – MMD3

Moreover, recessive *ANO5*-mutations were linked to a neuromuscular phenotype classified as a distal non-dysferlin Miyoshi myopathy (MMD3) in different publications.[13–15] Patients present with distal weakness and reduced muscle strength, especially affecting the calf muscles. Reduced strength in knee flexion (mediated by biceps femoris, semitendinosus and semimembranosus commonly known as "hamstring group") has been reported

Table 2 Clinical data of 20 patients with confirmed genetically confirmed LGMDR12 (modified presentation of clinical data published by Hicks and co-workers[13]).

Clinical characteristics	Frequency
Male	18/20
Female	2/20
Adult onset (>20)	19/20
HyperCKemia	20/20
Muscle atrophyrowhead	19/20
Quadriceps/hamstring muscles	15/20
Calfs	14/20
Upper limbs (biceps, triceps, brachioradialis)	7/20
Muscle weaknessrowhead	20/20
Upper limbs	13/20
Lower limbs proximal	20/20
Mild	4/20
Moderate	3/20
Severe	13/20
Lower limbs distal	17/20
Mild	12/20
Moderate	4/20
Severe	1/20
Asymmetry	18/20
Myoglobinuria	3/20
Scapular winging	7/20
Functional statusrowhead	
Ambulant	16/20
Restricted	3/20
Severely restricted	1/20

as well. On rare occasion, involvement of the upper limb has been observed in form of reduced strength of elbow flexion. A distal weakness of the upper extremities has not been described thus far. Onset is commonly seen between the third and fifth decade.[1,9,11,15]

In brief:

- ➢ Distal weakness and reduced muscle strength especially affect the calf muscles.
- ➢ Onset is commonly seen between the third and fifth decade.

Further muscular complications

Anoctaminopathies are not necessarily manifesting with muscle wasting and it has been proven that at disease onset, often other symptoms such as hyperCKemia, myalgia or exercise intolerance can be present.[16] In this context, it is important to note that although myalgia and exercise intolerance are not commonly mentioned in publications summarizing the clinical findings of Anoctaminopathy-patients, these were observed as singular or in combination as symptoms of onset. Interestingly, it was observed that female patients more often present without muscular involvement assuming a less severe manifestation of Anoctaminopathies in this gender.[11]

Episodes of rhabdomyolysis in *ANO5*-patients have been reported in several papers[11,16–20]: in a cohort of 13 patients with genetically confirmed muscular dystrophy, *ANO5*-patients represent the second largest subcohort presenting with additional rhabdomyolysis (FKRP: $n = 6$, ANO5: $n = 3$; CAPN3: $n = 2$; DMD: $n = 2$).[19] Rhabdomyolysis is often preceded by years of elevated CK-values or myalgia and usually muscle weakness.[16,19] However, further clinical data suggest that rhabdomyolysis can precede such symptoms and can even be present in the initial stage of manifestation of pathogenic *ANO5*-mutations.[11,18,19] Notably, one study highlighted an increased risk of associated rhabdomyolysis in patients with amyloid depositions in the muscle biopsy (see below).[19]

These clinical findings suggest that *ANO5* should be considered as a candidate gene even if "only" one of the above-mentioned symptoms is present without further overt muscle symptoms such as manifest muscle weakness or -pain.[11,14–16,21]

In the context of variable clinical presentations, a molecular genetic study of Polish limb girdle muscular dystrophy patients, revealed that *ANO5*-patients harbored additional variants in other genes associated with muscle pathology, possibly affecting clinical presentation as well as the disease progress.[22]

In brief:

- ➢ Myalgia and exercise intolerance were observed as singular or in combination as symptoms of onset.
- ➢ Episodes of rhabdomyolysis in *ANO5*-patients have repeatedly been reported.
- ➢ Pathogenic variants in further genes associated with the manifestation of muscular diseases might impact on the clinical presentation.

Cardiac involvement

Since *ANO5* has been shown to be expressed in cardiac tissue and encodes a putative chloride channel, arrythmia and other cardiac abnormalities have been suspected to be part of the clinical presentation.[2,7,9,10] A variety of findings on cardiac dysfunction in *ANO5*-patients reveal multifaceted results: a study focusing on cardiac symptoms in 19 *ANO5*-patients revealed ventricular arrhythmia in one patient, left ventricle (LV) dilatation in two patients, one patient presented with LV dysfunction and combined dilatation and dysfunction was moreover reported in two further patients thus fulfilling the criteria of a dilated cardiomyopathy. Consequently, the authors postulated that dilative cardiomyopathy can occur as a possible clinical complication in *ANO5*-patients.[23] Another study on a cohort of 20 *ANO5*-patients showed that none of the patients presented with reduced LV-ejection fraction being set at $<55\%$. However, 16% of the patients showed high frequency of ventricular premature beats (which is two-fold higher compared to a study with 678 healthy age-matched controls). Here, the authors concluded that *ANO5*-patients display no signs of a structural heart disease, but may have an increased risk of ventricular arrhythmia.[9] In a further study, authors postulated that even if *ANO5*-patients are "still" free of muscle weakness, they can present with moderate cardiac complications and may benefit from regular cardiac follow-up.[16] In a larger cohort of 52 patients (LGMDR12 and MMD3), LV-dysfunction was identified in five and cardiac arrhythmia in two cases.[17] Taken together, these studies show that cardiac abnormalities can be part of the phenotype caused by recessive *ANO5* mutations and cardiac examination should thus be recommended in patients with diagnosed or supposed *ANO5*-related muscular disorder. A cohort of 10 *ANO5*-patients underwent cardiac examination by MRI. Six patients presented with abnormalities detected in cardiac MRI, despite being clinically asymptomatic in relation to the cardiac function. The spectrum of pathologies observed included reduced left ventricular ejection fraction, mild left ventricular hypertrophy and left atrial dilation, which can be suggestive for an early-stage dilative cardiomyopathy, a finding which had been described in *ANO5*-patients before (see above).[24]

In brief:

- ➢ Dilative cardiomyopathy may occur as a possible clinical complication in *ANO5*-patients.
- ➢ *ANO5*-patients may have an increased risk of ventricular arrhythmia.
- ➢ Cardiac examination should be recommended in patients with a diagnosed or supposed *ANO5*-related muscular disorder.

Pulmonary involvement

So far, no pulmonary peculiarities including ventilatory failure or nocturnal hypoventilation have been identified in *ANO5*-patients.[7,9,11,21,25,26]

Typical features of skeletal muscle MRI in *ANO5*-patients

Several muscle MRI-studies have been performed to evaluate changes in *ANO5*-patients.[27] They all describe a

characteristic pattern of muscle involvement, with predominant fatty degeneration of the medial gastrocnemius and adductor magnus muscle.[8,13,15,16,27] Additionally, semitendinosus, semimembranosus and quadriceps muscles are involved, and in some cases also slightly the gracilis, sartorius, soleus and tensor fasciae latae muscles.[8,13,27] During disease progress, the biceps brachii muscles were also described to degenerate.[27] Moreover, muscle edema could be displayed by STIR sequences in the above-mentioned muscles, especially early in the disease course, preceding fatty degeneration. Later in the disease, muscle tissue edema decreases.[27] Although this pattern of muscle involvement is typical for *ANO5*-patients, there is great variability in the degree of degeneration, even among LGMDR12 siblings.[15] In contrast to other myopathies, MRI changes are often asymmetric.[8,13]

In brief:

- ➢ Typical muscle MRI findings include fatty degeneration affecting different muscle including medial gastrocnemius and adductor magnus muscle.
- ➢ Muscle edema might be present especially early in the disease course, preceeding fatty degeneration.
- ➢ MRI pathologies are often asymmetric.

Muscle biopsy findings

Same as for other forms of muscular dystrophies, pathological findings are common in biopsies from *ANO5*-patients: a cohort of 38 patients with *ANO5*-mutations was sub-classified into group 1 ($n = 20$) showing muscle weakness, which was further subdivided into an LGMDR12 and an MMD3 subcohort, and group 2 ($n = 18$) having hyperCKemia or being asymptomatic.[16] Within group 1, 18 patients underwent a muscle biopsy followed by histological analysis: 13 of these (72%) showing abnormal biopsy findings including "dystrophic features" such as muscle fiber necrosis, increase of endomysial fibrous tissue and regeneration as well as "myopathic features" such as variation in fiber size and internalization of nuclei. Within group 2, 15 patients received a biopsy followed by histological analysis and in 14 (93%) the abnormal biopsy findings mentioned above were detected.

Commonly myopathic/dystrophic changes of affected skeletal muscle tissues can be identified at various degrees. Often myonuclei may appear in pycnotic clumps and an increased amount of internalized myonuclei can be observed.[8,11,13,18,28] A publication on a woman with exertion-induced myalgia and weakness in the hip girdle (manifesting at the age of 40) and constantly elevated CK values, reported on a necrotizing myopathy based on the histological examination. Remarkably, later the patient was diagnosed with recessive homozygous c.191dupA (exon 5) mutation.[29] This was the first report mentioning a necrotizing myopathy as the predominant histological feature and therefore extends the histological spectrum of Anoctaminopathies. In addition, changes of the connective tissue are reported in form of endomysial fibrosis, which was previously held as distinctive feature to other dystrophies.[8] Milone and co-workers reported on amyloid deposition in biopsies of *ANO5*-patients based on the identification of congophilic deposits within blood vessel walls and around muscle fibers. However, subtyping of those amyloid depositions failed.[18] Nevertheless, in the context of a further study, Apolipoprotein A1, Apolipoprotein A4, Apolipoprotein E, Gelsolin and Serum Amyloid P component were detected when performing amyloid subtyping.[10]

Recently, Seguí and co-workers[30] published clinical, pathological and molecular findings of three unrelated *ANO5*-patients: all three presented with a different muscular phenotype — each with one of the already described clinical presentations (LGMDR12, distal myopathy, asymptomatic hyperCKemia). Examination of muscle biopsies revealed in one patient the presence of non-specific myopathic changes such as mild necrosis and regenerating fibers with internalization of some myonuclei. The second patient showed no myopathic changes except for mild accumulation of lipid droplets. The third patient presented with many ragged-red fibers, endomysial inflammation, nuclei internalization, partial invasion of muscle cells and necrosis. Of note, the latter pathological muscle findings have not been described before in *ANO5*-patients and are usually observed in mitochondrial myopathies such as MERRF syndrome. Based on this novel finding, mitochondrial studies were performed revealing a very mild complex III deficiency. Of note, no changes were found in the mitochondrial DNA exome sequencing confirmed the c.191dupA and c.692G>T *ANO5*-variants and excluded variants in other neuromuscular genes. Hence, the pathological findings observed in the third patient enlarge the current knowledge of histological findings for *ANO5*-related muscle diseases and indicate mitochondrial myopathic changes in *ANO5*-patients. Along this line, another microscopic study focusing on findings in muscle biopsy specimen derived from *ANO5*-patients identified subsarcolemmal mitochondrial proliferation using modified Gömöri Trichrome staining and oxidative reactions in approximately 70% of fibers. Subsarcolemmal mitochondrial proliferation was also confirmed by electron microscopy.[21] However, it remains to be elucidated whether mitochondrial abnormalities are a common feature associated with recessive *ANO5*-mutations.

An electron microscopic study on a Finnish family with confirmed *ANO5*-mutations and a distal phenotype showed disruption of sarcolemmal membranes, but intact myofibrillar architecture and absence of subsarcolemmal accumulation of vesicles.[15]

In brief:

- ➢ Muscle biopsies of *ANO5*-patients show "dystrophic features" including muscle fiber necrosis, increase of endomysial fibrous tissue and regeneration → features can present of varying degree of severity.
- ➢ Muscle biopsies of *ANO5*-patients show "myopathic features" including variation in fiber size, internalization of nuclei and mild increase of lipid droplets → features can present of varying degree of severity.
- ➢ Mitochondrial pathology was occasionally described in muscle biopsies of *ANO5*-patients, but more detailed studies are needed to further elucidate this related pathophysiology.

➢ For the cataloging of ultra-structural pathologies, more electron microscopic studies on muscle biopsy specimen derived from *ANO5*-patients are needed.

Gender differences

Multiple studies highlighted a male predominance for the *ANO5*-related muscular disorders and moreover postulated that women are showing a less severe phenotype.[2,8,9,13,25] So far, no clinical or biochemical explanation for this phenomenon exists and one might speculate that the hormone status or the efficient expression of X-chromosomal modifiers (as already identified for spinal muscular atrophy[31]) may influence clinical manifestations. Consequently, women might be underrepresented due to recruitment bias excluding them from the large cohort studies. Notably, two asymptomatic women were identified carrying the mutation after diagnosing recessive *ANO5*-mutations in their symptomatic brother.[25] However severe progression has been seen in female patients as well, as reported in 2017 for a female patient showing signs of weakness at the age of 22.[11]

In brief:

➢ Males are predominantly affected → reason for that phenomenon is unknown.
➢ Non manifesting female carriers were also reported.

Molecular genetic findings

Since phenotypic appearance of *ANO5*-related muscle diseases show a considerable variability and many "Anoctaminopathy features" are also characteristic for other muscular disorders, genetic testing is recommended for approaching the correct diagnosis. Molecular genetic testing can be performed by various approaches including direct Sanger sequencing, multigene panels, both should inquire the most common variations of the genetic mutational spectrum of the given area[32] or unbiased next generation sequencing (whole exome or whole genome). With the start of genetic research on Anoctaminopathies, a considerable number of pathogenic and putative pathogenic variants were detected. The *ANO5*-gene consists of 22 exons and pathogenic variants seem to be evenly distributed throughout the gene.[8,9,11,14,25,29,33] Notably, some exons can harbor more than one pathogenic mutation and therefore might be more susceptible to DNA-changes.[14] Deletions and insertions within the *ANO5*-gene were also reported[21,34] as well as missense mutations.[7,25,26,34,35] Interestingly, missense variants seem to be the most frequent form of pathogenic DNA-changes followed by nonsense mutations.[7,17,25,26,34] Other forms of mutations such as frameshift, synonymous, splice site or intronic mutation are less frequently reported.[7,25] In Table 5, we summarize the most common mutations, their prevalence as well as geographical distribution.

As presented in Table 5, the most common pathogenic variants are c.191dupA (exon 5) and c.2272C_T (exon 20),[7] whereby the c.191dupA variant was even confirmed as a founder mutation[13]: a SNP in exon 10 and a microsatellite marker 135 kb downstream of *ANO5* were investigated in mutant alleles and normal Northern European control chromosomes. All mutant alleles were positive for the SNP and 19/22 were positive for the microsatellite marker, whereas only 13% of the control chromosomes carried the SNP and 24% carried the microsatellite marker from the control chromosomes. In addition, a Fisher's test indicated a strong linkage disequilibrium between the mutation and the two chosen polymorphisms ($P < 0.0001$). The c.191dupA mutation is considered to be the most common pathogenic variant within populations originating from Northern Europe and has been detected in 51 out of 84 (61%) mutated alleles in a cohort of German and British Origin with two *ANO5*-mutations.[25]

Bolduc et al identified a variant in exon 20 in a Finnish family (c.2272C>T), resulting in a substitution from a conserved arginine to a cysteine residue.[1] Screening for this variant in French–Canadian families and families from the United Kingdom failed to detect this variant. However, a research group[14] from Finland screened a total of 25 patients and detected 11 different *ANO5* variants of which 8 were described before. Of note, the most frequent variant present in 20/25 patients was the heterozygous c.2272C>T (p.R758C). Due to the high frequence within the Finnish population, this pathogenic was first considered to be a founder mutation. Nevertheless, based on additional clinical and molecular genetic research, this previous assumption was excluded and this variant is now considered to be of central European origin.[13] To date, no further variants were confirmed or suspected to be a founder mutation.

A report of the first large cohort study from Southern America included a total of 37 patients from 34 non-related families in Brasil. Most of the *ANO5*-patients where white (86.5%) and harbored the variants c.191dupA, c.2272C>T, c.692G>T, commonly detected in European cohorts. This molecular genetic observation is most likely due to a European ancestral background from the 16th and 17th century.

The first publication of a *ANO5*-family from China reported on the c.220C>T *ANO5*-variant, which shifts arginine into a stop codon (in the 74th codon), resulting in a termination of protein translation.[8] The clinical and genomic evaluation of 207 cases suffering from myopathies in India allowed to draw a molecular genetic diagnosis in 101 patients (49%) utilizing exome sequencing. Among these cases, 3 (3%) presented with pathogenic *ANO5*-variants: the homozygous c.1406G>A nucleotide exchange in two patients and the homozygous frameshift variant c.2141_2144dupCTCA in the third case.[36]

Given that molecular genetic testing often results in the identification of ambiguous variants, valuable tools enabling the testing of pathogenicity are required. For *ANO5*, Jarmula and co-workers demonstrated that application of *in silico* tools are suitable to evaluate damaging effects of ANO5-variants classifying such as likely pathogenic according to criteria of the American College of Medical Genetics and Genomics (ACMG). In addition, molecular modeling of mutations allowed to highlight substantial changes in ANO5 conformation that could affect the protein structure and function and thus provide further information regarding the potential pathogenicity of *ANO5*-variants.[22]

In brief:

- ➢ The c.191dupA variant (in exon 5) and the c.2272C_T (exon 20) variant are the most frequent pathogenic *ANO5* mutations.
- ➢ The c.191dupA variant is a founder mutation.
- ➢ *In silico* tools were generated to test the pathogenicity of ambiguous *ANO5* variants.

Genotype—phenotype correlations

Multiple studies focused on a potential correlation between genotype and phenotype.[7,13,14,25,37] No correlations were found for the most frequent mutations (c.2272C_T and c.191dupA) - both were associated with a broad spectrum of phenotypic appearance and no phenotype was restricted to a particular mutational pattern.[14] Additionally, no correlation was detected in patients being compound heterozygous for the c.191dupA mutation and a second pathogenic variant.[13] However, one clinical report postulated that the c.191dupA is associated with a milder phenotype and later onset of the disease. Nevertheless, the authors also acknowledge that a larger number of patients would be needed to draw a statistically significant conclusion.[25]

A further study reported that different mutations might lead to different biochemical alterations of the ANO5 protein in turn resulting in the clinical manifestation of different muscle pathologies. However, more precise biochemical data for each of the reported pathogenic variants would be crucial to support this assumption.[37] Hence, since the *ANO5*-related clinical profile is much more diverse and new muscular phenotypes are detected continuously, to date no clear correlation has been reported. Same observations were already made for "Dysferlinopathies" and "Caveolinopathies", caused by recessive mutations in *DYSF* and *CAV3*, respectively.

In brief:

- ➢ Previous studies are not indicative for the existence of clear genotype—phenotype correlations.
- ➢ Pathogenic biochemical alterations of the ANO5-protein might impact on different clinical presentations but further combined clinical and pre-clinical studies are needed to support this assumption.
- ➢ LGMDR12 and MMD3 are genetically not distinguishable.

Own studies

Skeletal muscle MRI findings in a cohort of four *ANO5*-patients

Here we investigated 4 patients with a compound-heterozygous *ANO5*-mutations by muscle-MRI (Table 3 and Fig. 2). Basically, findings in our LGMDR12 patients were in line with current knowledge about affected *ANO5*-muscles. All patients had fatty degeneration of the medial gastrocnemius muscles. Two patients also showed involvement of other calf muscles, except for the anterior compartment, which was always spared in our patients (Table 3 and Fig. 2). Regarding thigh muscles, the adductor magnus showed fatty degeneration in all patients of various degrees. Patient 2 and 3, and to a much lesser extent patient 4, also showed involvement of the other ischiocrural muscles, esp. semimembranosus, semitendinosus and biceps femoris muscles as well as parts of the quadriceps muscle (esp. patient 3). Patient 3 also had fatty degeneration of the left gracilis. Thus, the pattern of fatty muscle degeneration is well in line with the *ANO5*-patients described previously.[8,13,15,16,27] Of note, patient 1 had general reticular fatty degeneration throughout the leg muscles apart from the more severely affected adductor and medial gastrocnemius muscles, and remarkably no visible progress after 10 years follow-up (Fig. 2). This has not been described in previous studies and may be related to so far unknown compensatory mechanisms which stabilize the disease course. This patient did not show any myoedema in both MRI scans. Myoedema were found in patients 2—4, who were investigated at an early time point of their *ANO5*-disease. Those findings are supported by the study of Ref. 27, who depicts a typical disease course with three periods of *ANO5*-associated myopathy: early (<10y disease duration (DD)), intermediate (10—20y DD) and late (>20y DD) period. According to his study, muscle edema is rather typical for the early and intermediate period, and decrease in the late period.[27] Our patients were well in line with those findings and also show asymmetric muscle involvement characteristically seen in *ANO5*-disease. Finally, our data also showed that the female patient was least affected, as had been described by Refs. 8,38.

Our conclusions: three aspects are crucial regarding muscle-MRI for clinicians to early recognize and diagnose a possible *ANO5*-associated myopathy: *(i)* the typical muscle involvement (esp. medial gastrocnemius, adductor magnus, semimembranosus and semitendinosus, later the biceps brachii muscles) with edema in early and increasing fatty degeneration during later disease course. Usually, there is no or late fatty degeneration of the gracilis and sartorius muscles. *(ii)* The asymmetric pattern of muscle involvement, and *(iii)* interindividual variability of disease course and muscle involvement, especially the fact that females are less affected than males.

Muscle biopsy findings in a cohort of six *ANO5*-patients

To elucidate inflammatory processes in the disease-cause of *ANO5*-related muscle disorders, a histological study was performed on a cohort of six genetically confirmed patients (Table 4). Hematoxylin and eosin (H&E) staining and Gömöri trichrome staining revealed a focal accumulation of necrotic fibers, mild fiber size variances as well as myophagocytosis. Endomysial fibrosis is minimal. NADH-TR staining showed a regular distribution of type I and type II myofibres with fine granularity. Alkaline phosphatase is only positive on some endomysial capillaries, while the perimysial connective tissue is not stained. However, Utrophin staining, as well as Laminin-α5 show up-regulation in regenerating fibers. MHC class I shows only single fibers with a sarcolemmal lining, while complement deposition is not detectable on the sarcolemma of healthy myofibres (note, the necrotic fibers, which accumulate C5b-9 non-specifically on the sarcoplasm) and not on any capillaries. MHC class II is negative (not shown) and CD45 as well as CD8 positive leukocytes and lymphocytes are diffusely distributed in the endomysium. $CD68^{+}/CD206^{+}$ macrophages accumulate in fibers showing myophagocytosis, while B cells are absent (not shown). CD56 weakly stains some

Table 3 Summary of the clinical, genetic, laboratory and radiological data from our patients with *ANO5*-associated myopathy.

	Patient 1 (A1/A2)	Patient 2 (B)	Patient 3 (C)	Patient 4 (D)
Current age (years)	62 y	61 y	57 y	56 y
Age at onset (years)	39 y	Unknown	36 y	48 y
ANO5 mutation	c.191dup (p.Asn64Lysfs*15) c.2272C>T (p.Arg758Cys)	c.191dup (p.Asn64Lysfs*15) c.155A>G (p.Asn52Ser)	c.191dup (p.Asn64Lysfs*15) c.2521-1delG	c.191dup (p.Asn64Lysfs*15) c.1733T>C (p.Phe578Ser)
CK max (U/L)	2581 U/l	2943 U/l	3524 U/l	4577 U/l
Age at MRI-examination (years)	48 y (A1) and 58 y (A2) (9 y and 19 y after disease onset)	58 (B) (disease onset unknown)	39 (C) (3y after disease onset)	50 y (D) (2y after disease onset)
Muscle MRI T1w findings	Fatty asymmetric degeneration of the right adductor magnus and both medial gastrocnemius muscles (A1). After 10 y progress of the fatty degeneration of the right adductor magnus. No further muscle involvement (A2).	Fatty, slightly asymmetric degeneration of the ischiocrural muscles, the right adductor magnus and rectus femoris, medial gastrocnemius and less the soleus (B).	Fatty asymmetric degeneration of the ischiocrural muscles, adductor magnus and longus, left gracilis, vastus lateralis, intermedius and medialis, asymmetric fatty degeneration of triceps surae (C).	Asymmetric fatty degeneration of adductor magnus, slightly right semimembranosus, left biceps femoris and left medial gastrocnemius muscles (D).
Muscle MRI STIR findings	No edema in the thigh and calf muscles (A1/A2).	Edema of the ischiocrural muscles, adductor magnus bilateral, right rectus femoris, asymmetric edema of soleus muscles (B).	Asymmetric edema of the quadriceps femoris, adductors and gracilis and the triceps surae (C).	Slight edema of the right adductor longus and soleus (D).

y = years; the term ischiocrural muscles comprises the semimembranosus, semitendinosus and biceps femoris muscles; the term triceps surae comprises the medial and lateral gastrocnemius and soleus muscles.

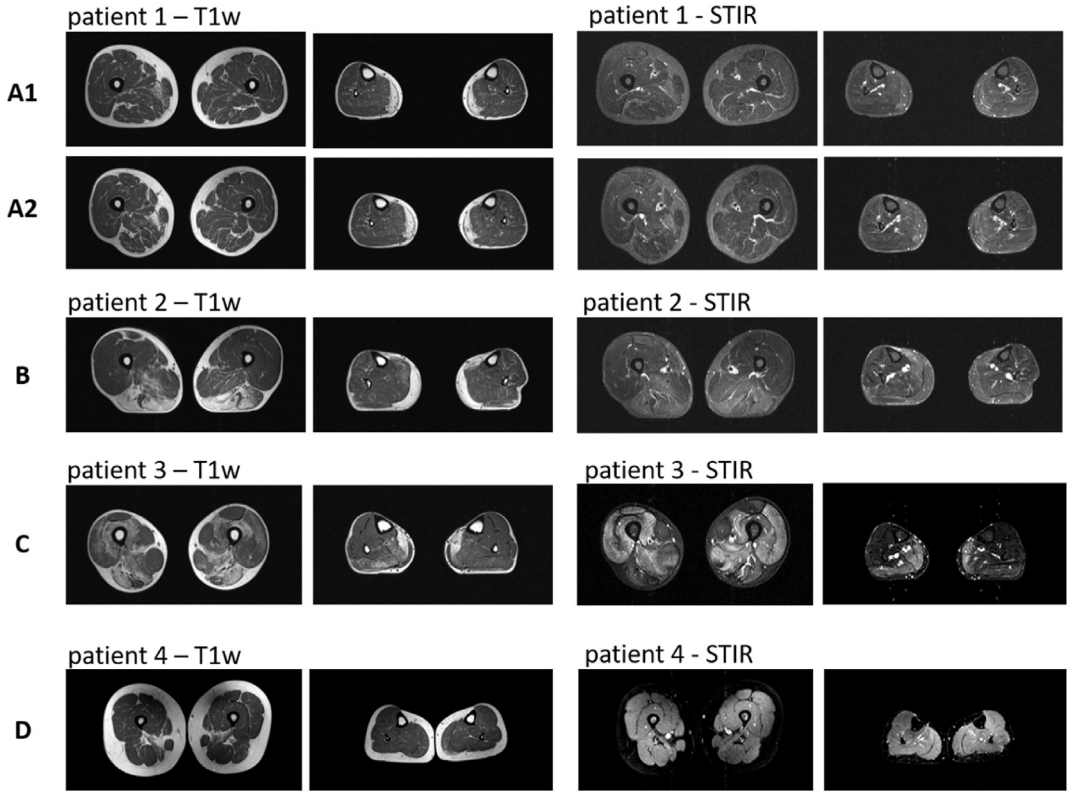

Figure 2 Skeletal muscle MRI of 4 *ANO5*-patients **(A—D)**. The two left panels show fatty degeneration in T1w-sequences, the two right panels display tissue edema in the STIR-sequences. Patient 1 (A1 and A2) has been investigated twice: at the time of first presentation (A1) and at follow-up after 10 years (A2). From each patient, representative images of thighs (left image in panel) and calfs (right image in panel) were chosen. STIR: short-tau-inversion-recovery-sequence.

Table 4 Summary of inflammatory findings in a cohort of six patients with genetically proven "Anoctaminopathy".

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
ANO5-mutation	Exon 7: c.364-2A>G	Exon 5: c.191dupA Exon 18: c.2000T>C	Exon 5: c.191dupA Exon 18: c.2000T>C	Exon 13: c.1210C>T Exon 20: c.2387C>T	c.2556G>A; c.1898+1G>A	c.191dupA; c.2521-1delG
Gender	Male	Male	Female	Male	Female	Male
Age at biopsy [years]	12	14	16	39	39	39
Necrosis	No	No	No	Yes	Yes	No
Fibres	Normal	FSV, internalized myonuclei	FSV, internalized myonuclei	FSV, internalized myonuclei	FSV, internalized myonuclei	Normal
MHC-cl. I	0	1	0	1	1	0
MHC-cl. II	0	0	0	0	0	0
CD68	0	1	1	1	2	1
CD45	0	1	0	2	1	1
CD8	0	1	1	1	1	1
C5b9	0	0	0	0	Sarc	Sarc
p62	0	0	0	0	0	0

FSV: fiber size variation; sarc: sarcolemmal.

Table 5 Summary of most frequent pathogenic variants in *ANO5* in different studies and entities.

Country	Prevalence of *ANO5* confirmed patients (LGMDR12)	Overall cohort	Most frequent *ANO5*-mutation	Study
Saudi Arabia	3%	Number of patients included is not mentioned (unclassified LGMD)	Not reported	29
Denmark	11%	40 patients (unclassified LGMD)	c.191dupA	2
Italy	2%	228 patients (unclassified LGMD)	c.1627dupA (Exon 15)	30
Finland	25%	101 patients (unclassified LGMD + calf distal myopathy + CK values > 2000 IU/L)	c.2272C_T	3
UK/Germany	25%	205 patients (undiagnosed with clinical suspicion of Anoctaminopathy)	c.191dupA	22
US	7%	4656 patients (undiagnosed with clinical suspicion of Anoctaminopathy)	c.191dupA	31

fibers while necrotic fibers are non-specifically stained. Markers of autophagy like p62 and LC3 were negative in all patients (p62 non-specifically stained in one patient in one area). Results of this histological study are shown for a representative case in Figure 3 and summarized in Table 4.

Our conclusions: As mentioned above muscular changes in *ANO5*-patients are mild and we could confirm these findings in our cohort. Muscle regeneration is accompanied by increased protein abundances of Utrophin and Laminin-α5. This is the first systematic investigation on *ANO5*-associated inflammatory processes revealing mild inflammatory processes which are characterized by CD45 and CD8 positive leukocytes in addition to CD68 and CD206 positive macrophages accumulating within myofibres showing myophagocytosis. Moreover, CD56 weakly stains some fibers.

Diagnostic work-up of *ANO5*-patients

Detection of certain protein deficiency or decrease by utilizing specific antibodies targeting the proteins of interest is a main tool in diagnostic step in patients suffering from muscular dystrophies, as reported for diagnosis of "Dysferlinopathy"[39] or "Caveolinopathy"[40] for example. A research team developed the first efficient antibody for detection of ANO5-protein deficiency by immunoblotting.[37] Antibody validation included *in vitro* experiments: COS-1 cells were transfected with hANO5wt-V5 (untransfected COS-1 cells were used as negative controls) and immunoblotting studies utilizing the ANO5-antibody (ANO5 N421A/85) and a V5 antibody revealed a band migrating just above the 100 kDa marker. Additionally, a protein signal was detected above >200 kDa, most likely representing ANO5-dimers. Along this line, they aimed to specify ANO5-protein expression in human skeletal muscle and to investigate if the ANO5-levels are dysregulated by different mutations: a protein band at approximately 100 kDa was observed in control muscle protein extracts and this band seemed to be reduced in the *ANO5*-patient samples. Due to several bands of different molecular weight, they performed tissue fractionation and assessed the bands in soluble, membranous and insoluble fractions in both, the control and *ANO5*-cohort. After quantification of the ANO5-protein level for the different bands in both cohorts, the authors concluded that ANO5 was clearly decreased in all *ANO5*-patients. Regarding the expression analysis of ANO5 across different muscles, no strong variation in level was detected between different investigated muscle groups (gastrocnemius, tibialis anterior, biceps femoris and semimembranosus). Immunohistochemical experiments with the antibody on human biopsy specimen were not yet successful. In addition, one other commercial and two costume made ANO5-antibodies were tested, but those three failed to recognize ANO5 in the immunoblot analysis. Taken together, these results suggest that immunoblot-based analysis of ANO5-protein level might be possible in the biochemical routine diagnostic work-up of these patients.

In brief:

- A first ANO5-antibody enabling to differentiate between patients and controls based on protein abundance is established since 2018.
- This antibody is not suitable for immunostaining approaches.
- Further studies utilizing muscle protein extracts derived from other patients suffering from muscular diseases are needed to address the potential reduction of ANO5-level as a secondary pathophysiological phenomenon.

Studies of ANO5-disease models

Muscular dystrophies are under constant research and to date the ones with mutations in genes encoding for the dystrophin-associated protein complex are the ones we know most about. These mutations lead to membrane fragility associated with the loss of sarcolemmal-cytoskeleton.[41] Other genetic subtypes of muscular dystrophies are associated with the defect of sarcolemmal repair (LGMDR2 & "Caveolinopathies"). The sarcolemmal membrane is under constant stress during contraction and therefore requires repair. This process relies on the fusion of membrane vesicle and two processes are known. Small patches are resealed by synthesis of new sarcolemmal membrane, large patches are usually sealed by satellite cells which proliferate and then undergo differentiation into myocytes. Based on the clinical overlap of "Anoctaminopathies" and LGMDR2 the hypothesis has been

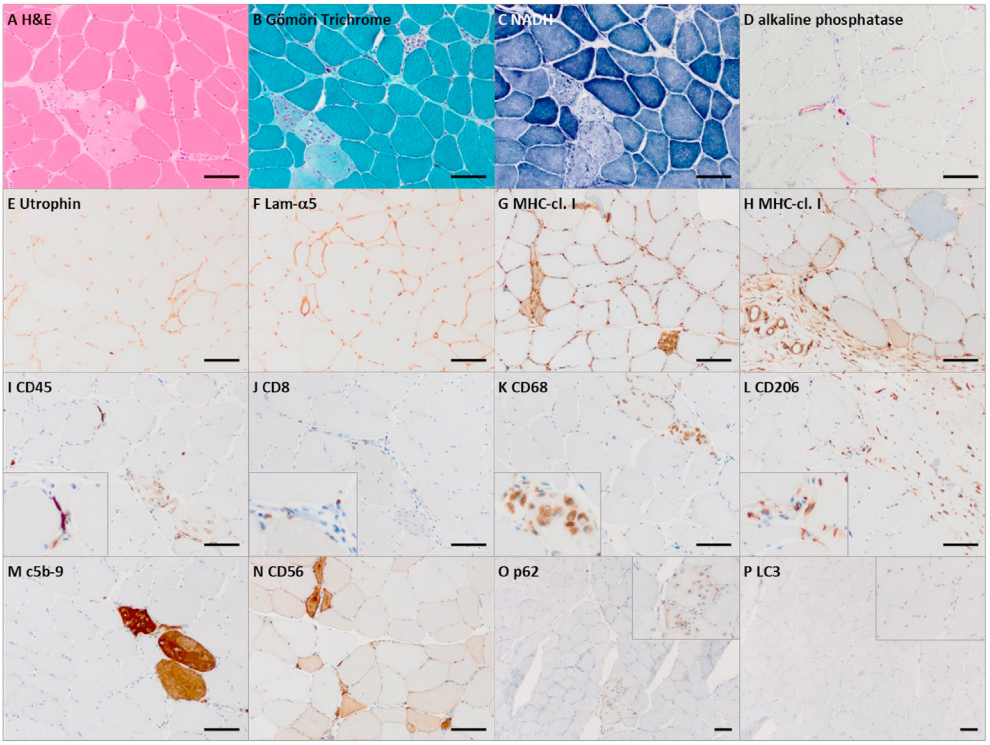

Figure 3 Histological representation of ANO5 patients. **(A)** H&E and **(B)** Gömöri trichrome showed necrotic fibers, mild fiber size variations and myophagocytosis. **(C)** NADH staining shows normal distribution of type I and type II myofibres. **(D)** Alkaline phosphatase is negative, except physiologically on endomysial capillaries. **(E)** Utrophin staining, as well as **(F)** Laminin-α5 show upregulation in regenerating fibers. **(G, H)** MHC-cl. I is positive on single fibers. **(I)** $CD45^+$ and **(J)** $CD8^+$ leukocytes/lymphocytes are diffusely distributed, while **(K, L)** $CD68^+/CD206^+$ macrophages accumulate. **(M)** Complement deposition is not detectable. **(N)** CD56 stains few fibers. Markers of autophagy like O: p62 and P: LC3 are negative. Scale bar = 100 μm.

gathered that pathogenic variants within the *ANO5*-gene may also lead to attenuated repair of the sarcolemma. To proof this hypothesis and to elucidate the underlying pathomechanisms underlying in *ANO5*-related muscular diseases on a general note, multiple disease models have been established and investigated.

Animal models

The first *Ano5*-knockout mouse model has been established by a research group from the United States using a C57BL/6J mice strain which were backcrossed for six generations before being used in their experiments.[4] After confirming lack of *Ano5*-expression via RT-PCR, contractile response in *Ano5*-knockout mice was analyzed as lack of force and an increased susceptibility to contractile damage are features commonly attributed to muscular dystrophies. However, no significant difference in *ex vivo* assessed muscle maximal force — when subjected to twitch contractions — was detectable between the mutant and wildtype animals. Subjecting the muscles from mutant and wildtype mice to lengthening contractions and again no distinguishable force deficit was noticed. In addition, *Ano5*-knockout mice developed a muscle mass of tibialis anterior and gastrocnemius in relation to their body weight similar to the ones of wildtype mice. Along this line, no obvious histopathological features were identified in *Ano5*-knockout mice at different ages by histological examination (H&E staining). Furthermore, they evaluated whether *Ano5*-defiency leads to alterations in the expression of proteins belonging to the Dystrophin—glycoprotein-complex including Dysferlin: immunofluorescence staining and immunoblot analyses on skeletal muscles did not reveal abnormalities in expression or localization. Furthermore, the quantitative level of the other anoctamins was investigated by applying quantitate RT-PCR. Also, this analysis did not show significant transcriptional upregulation of the other anoctamins. This result was confirmed on the protein level by immunoblot studies. Given that *ANO5*/*Ano5* is also expressed in cardiac tissue[2,9,10] potential cardiac involvement was evaluated but no major differences in cardiac functions or the thickness of the interventricular septum was detected. After two weeks of isoproterenol application *Ano5*-knockout mice developed similar left ventricular dilation as wildtype mice. Compared to wildtype animals, *Ano5*-knockout mice also did not show significant perturbations in the regenerative capabilities of muscles after cardiotoxin induced injury. Based on the combined findings, the authors concluded that disruption of *Ano5*-expression in mice does not cause overt muscle histopathology.

Another research group[41] created a further *Ano5*-knockout mouse model and *Ano5* knockout was confirmed by RT-PCR genotypically with a reduction of *Ano*5-transcript post cassette of >99%. To scale Ano5 protein abundance, muscle lysates were analyzed on under non denaturing

conditions by blue native gel electrophoresis: no protein signal was detected at approximately 420 kDa in the mutant animals — surprisingly, the signal was greatly reduced in muscles derived from heterozygous mice as well. Phenotyping of these animals led to data being controversy to the one described above by displaying many features which are also represented in Anoctaminopathy patients including hyperCKemia, variable muscle weakness, diminished muscle force, altered muscle fiber diameter, exercise intolerance. Initiation of membrane damage by intense laser pulse in isolated flexor digitorum brevis and subsequent quantification of FMI1-43 (a thoroughly used dye to studying membrane repair) *Ano5*-knockout fibers presented with increased FMI1-43 compared to control fibers: in the wildtype fibers, the fluoresence leveled off after 190 s, whereas in *Ano5*-deficient fibers it kept increased for the duration of the experiment. Of note, overexpression of exogenous *Ano5* cDNA (using adeno-associated virus) in *Ano5*-knockout fibers partly restored membrane resealing. In addition, it was examined if Ano5-deficiency interferes with capacity of muscles to regenerate after injury induced by the application of cardiotoxin. In the wildtype mice, muscles regenerated within one month (with central nuclei being left as sign of newly regenerated muscle fibers), whereas in Ano5-deficient muscles, after three months the mean fiber diameter still was significantly reduced and the number of central nuclei exceeded the number observed in wildtype animals. Additionally, the muscle morphology was changed in the *Ano5*-deficient mice with a larger number of fibers being short and/or exhibiting an ovoid shape and displaying a smaller number of nuclei compared to the muscles fibers of wildtype animals. To sum up, phenotyping of this second mouse model proved that loss of *Ano5*-expression in mice leads to similar muscular perturbations compared to loss of functional ANO5 in humans including dysfunction in muscle membrane repair and regeneration. Additionally, phenotyping of the mouse model revealed the presence of aggregates in muscle cells of mutant animals which have not been described in human Anoctaminopathy patients yet. These aggregates are suggested to be related to disrupted Ca^{2+} signaling, but exact knowledge is still missing.

Further functional studies on *Ano5*-knockout mice were performed to analyze membrane repair deficits: accumulation of several Annexin-proteins at the side of injury was observed, which aggregate to form a cap at the site of injury in healthy muscle fiber. Annexin A2 levels were almost twice elevated in mutant fibers compared to such derived from control animals, while Annexin A6/A1/A5 levels where extensively inhibited in the *Ano5*-deficient mouse muscle. Thus, the underlying plasma membrane repair defect in *ANO5*-patients might be caused by the missing annexin coordination as observed in the murine model.[42]

The first *Ano5*-deficient rabbit model was introduced based on CRISPR-Cas9 approach.[43] Investigation of *Ano5*-knock-out by quantitative RT-PCR using three sets of primers revealed that only one primer showed reduced expression of *Ano5*-transcripts in the *Ano5*-knock-out rabbits. The other two showed similar transcript levels compared to control animals suggesting that the mutant *Ano5* transcripts are expressed at similar levels. Clinical and histopathological characteristics of Anoctaminopathies in human were assessed by studying increased percentage of centrally placed nuclei, scattered necrosis with inflammatory infiltratrates, fibrosis and fatty replacement in the muscle biopsies as well as elevated CK-levels. Additionally, as for the two above-mentioned mouse models, muscle regeneration was studied by the utilization of cardiotoxin (injected in the gastrocnemius muscles) and a profound increase in fibrosis with delayed regeneration accompanied by an increased number of smaller muscle fibers 14 days' post cardiotoxin injection was detected in mutant animals. Hence, the *Ano5*-KO rabbit model resembles many of the aspects seen human Anoctaminopathy patients. Therefore, the authors advocate this new model for ongoing research related to the field of Anoctaminopathies.

In brief:

- The first *Ano5*-knockout mouse model (C57BL/6J strain) did not show features of a muscular disease.
- Another *Ano5*-knockout mouse model (also C57BL/6J strain) presented with a muscular disease-phenotype on the functional, regenerative, and histological level.
- Further phenotyping of this mouse model revealed the presence of aggregates in muscle cells of mutant animals which have not been described in human Anoctaminopathy patients yet → these aggregates are suggested to be related to disrupted Ca^{2+} signaling, but exact knowledge is still missing, and further studies are needed to proof this assumption.
- The first *Ano5*-deficient rabbit model was introduced based on CRISPR-Cas9 approach and presented with many of the aspects seen human Anoctaminopathy patients.
- The animal models resembling the human phenotype might severe as valuable tools for the pre-clinical testing of therapeutic intervention concepts.

In vitro models

A research group performed an *in vitro* study utilizing C2C12 myoblasts to obtain a broader understanding of pathomechanisms underlying in the molecular genesis of *ANO5*-related muscle diseases. After silencing of *Ano5*-expression in C2C12 myoblasts by using the shRNA technique and confirming the efficiency by quantitative RT-PCR studies, the interference with myogenesis and E—C-coupling was addressed: *Ano5*/Ano5 expression pattern (mRNA + protein) during the period of differentiation in was measured in *Ano5*-silenced and control C2C12 myoblasts. During differentiation, in control cells, a gradual increase of *Ano5*-expression was noticed.[3] However, Ano5-deficiency did not result in lack of myotube formation confirmed by quantitative immunoblotting focusing on myogenic marker proteins (MyHC and myogenin).[3] Nevertheless, morphological differences were detected in Ano5-depleted C2C12 cells. Pathomorphological features included a generalized bigger and broader shape, clustered myonuclei; in control cells, a nuclear positioning defect was present in 16.4% while for the Ano5-depleted C2C12 cells, the percentage was 71.4% and the number of aligned nuclei was significantly reduced (Ano5-depleted 12.3%; controls 72%). Hence, Ano5-depletion does not

interfere with myoblast differentiation but impacts on proper nuclear positioning in C2C12 myoblasts.[3] Further studies toward a broader understanding of the nuclear position defect assessed Kif5b, a nuclear motor protein. While Kif5b increased during differentiation in the control cells, its expression remained constant in the Ano5-depleted C2C12 cells. Microtubule distribution such as alpha- and beta-tubulins was not altered as confirmed by immunostaining. To proof if Ano5-depletion interferes with E–C-coupling, Ca^{2+} signaling in myotubes was investigated. Measured depolarization Ca^{2+} transients were 30% smaller in response to 100 mM K^{+} solution in Ano5-depleted myotubes. During rest Ca^{2+} transients were equal in both. Further experiments revealed that the reduced amount of Ca^{2+} during depolarization is based on a decreased amount of Ca^{2+} stored within the sarcoplasmic reticulum (SR). Given that the impaired capacity of Ca^{2+} storage within the SR in Ano5-depleted cells might be caused by an insufficient function of the SERCA pump (responsible for Ca^{2+} uptake into the SR), protein abundances of SERCA and DHPR were studied along with the expression of RYR1, calsequestrin-1 and junctophilin-2, which also represent important key players in E–C-coupling. While SERCA and DHPR expression were reduced, RYR1, calsequestrin-1 and junctophilin-2 remained unchanged. Additionally, the co-localization of DHPR and RYR1, necessary for the mechanical coupling between each other was diminished in Ano5-depleted myotubes down to 84% compared to the control cells. Taken together, E–C-coupling in Ano5-depleted myotubes seems to be reduced due to decreased expression of DHPR and SERCA and perturbed DHPR–RYR1-coupling.

In 2019, Chandra and co-workers published their functional work focusing on plasma membrane repair and myogenesis on a myoblast cell line obtained from a *ANO5*-patient with genetically confirmed (homozygous c.2272C>T mutation) MMD3.[44] Remarkably, the mutant cells revealed the same differentiation ability as control myoblasts. This finding was confirmed by assessing the number of myonuclei in myosin heavy chain 3 (MYH3)-positive myotubes after 10 days of differentiation, showing similar results for *ANO5*-mutant and control cells. Additionally, differentiation marker protein level (MYH3, desmin, alpha-actinin) were studied by immunoblot analyses and no significant differences between control and patient-derived cells were identified. Furthermore, the question was assessed whether ANO5-deficiency interferes with PMR (plasma membrane repair): myoblasts were injured utilizing glass beads and numbers of injured fibers were compared between the patient-derived and the control cells revealing a significant higher number in *ANO5*-mutant cells. In addition, membrane damage was induced by a laser assay following the determination of the time the lipophilic FM dye needs to enter the damaged cell until PMR was finished and this experiment revealed a prolonged "entry-duration" in muscle cells derived from the *ANO5*-patient compared to control cells. To further proof this molecular observation, transient transfection of ANO5-GFP in *ANO5*-patient derived cells was carried out resulting in enhanced PMR and consequently leading to similar time frame of membrane repair after focal injury in the mutant and control cell line, respectively. To confirm a physical subcellular localization of the exogenous ANO5-GFP, co-immunofluorescence studies with the luminal endoplasmatic reticulum marker RFP-KDEL were performed showing a localization of ANO5-GFP within the SR. The authors hypothesized that based on its subcellular localization and the data published, ANO5 may play a role in controlling cytosolic Ca^{2+} level and therefore in the maintenance of the SR integrity after damage of the ER membrane. This assumption was proven by showing that in control muscle cells, the amount of fragmented SR was about 10% after focal injury while in patient-derived cells the amount was nearly 50%. Along this line, patient-derived myoblast showed a 2-fold reduction in RFP-KDEL signal close to the plasma membrane in uninjured patient myoblasts in comparison to control myoblasts, assessed by internal reflection fluorescence microscopy. Summarizing, ANO5 seems to stabilize the plasma membrane proximity in resting cells and integrity in injured cells.

Schreiber and co-workers[45] tethered ANO5, -8, -9, -10 to non-lipid raft regions on the plasma membrane of HEK293 cells. Moreover, they observed an increase of anion permeability at the plasma membrane tethered by ANO-proteins, which induced by purinergic stimulation and increased intracellular Ca^{2+} concentration. Since scramblase activity had been reported for ANO5-proteins, furthermore scramblase activity was evaluated in HEK293 cells transfected with TMEM16-CFP-CAAX paralogs localizing to the plasma membrane: ionomycin-induced scrambling activity measured by staining of phosphatidylserine on the outer leaflet targeting Annexin-V was significant reduced in the ANO-CFP-CAAX overexpressing cell-line. In addition, P2X7-receptor-dependent-mediated membrane blebbing, which occurs in the presence of PtdSer expression (phosphatidylserine), was quantified showing diminished results for the HEK293 cells overexpressing ANO-paralogues including ANO5.

In brief:

- Ano5 silencing (shRNA-based) in C2C12 cells led to pathomorphological features including a generalized bigger and broader shape, clustered myonuclei and a myonuclear positioning defect accompanied by a general reduction of myonuclei and perturbed Kif5b (nuclear motor protein) expression.
- E–C-coupling in Ano5-depleted C2C12 myotubes seems to be reduced due to impaired capacity of Ca^{2+} storage within the SR, decreased expression of DHPR and SERCA and perturbed DHPR–RYR1-coupling.
- Studies on a myoblast cell line obtained from an *ANO5*-patient (homozygous c.2272C>T mutation; MMD3) revealed a perturbed capability of plasma membrane repair.
- Based on subcellular localization studies of recombinant ANO5 in human muscle cells, it was postulated that ANO5 may play a role in controlling cytosolic Ca^{2+} level and therefore in the maintenance of the SR integrity after damage of the ER membrane.

➢ ANO5 tethers to non-lipid raft regions on the plasma membrane of HEK293 cells and scrambling activity is significantly reduced and P2X7-receptor-dependent-mediated membrane blebbing is altered in ANO-CFP-CAAX overexpressing HEK293 cells.

Therapeutical approach

To date, no curative treatment for Anoctaminopathy patients is available. Over the past years, clinical data have been collected, enabling a better understanding of natural history and disease progression of LGMDR12/MMD3. Like other LGMDs, LGMDR12-patients are advised to avoid excessive exercise and prevent obesity, since both conditions are assumed to enhance disease progression. On the other hand, physiotherapy as well as moderate sports activity is recommended to avoid loss of mobility and prevent contractures.[11,12] Regarding drug therapy or medical treatment in LGMDR12, it is important to note that steroid treatment showed no improvement.[36] Although no specific treatment of LGMDR12 is currently available, a first animal model for the LGMDR12 has been created using CRISPR-technology.[43] This may be an important first step towards the pre-clinical testing of a novel therapeutic approaches such as innovative gene therapy.

In brief:

➢ Excessive exercise and obesity should be avoided.
➢ Physiotherapy and moderate sports activity are recommended.
➢ Steroid treatment showed no improvement.
➢ Therapy of LGMDR12 is currently restricted to symptomatic treatment.
➢ For disease-specific treatment, including novel gene therapies, further preclinical and clinical studies will have to be performed in the future.

Conflict of interests

Authors declare no conflict of interests.

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