RESEARCH ARTICLE

Cross-sectional study into age-related pathology of mouse models for limb girdle muscular dystrophy types 2D and 2F

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Abstract

Limb girdle muscular dystrophy (LGMD) types 2D and 2F are caused by mutations in the genes encoding for α- and δ-sarcoglycan, respectively, leading to progressive muscle weakness. Mouse models exist for LGMD2D (Sgca−/−) and 2F (Sgcd−/−). In a previous natural history study, we described the pathology in these mice at 34 weeks of age. However, the development of muscle pathology at younger ages has not been fully characterised yet. We therefore performed a study into age-related changes in muscle function and pathology by examining mice at different ages. From 4 weeks of age onwards, male mice were subjected to functional tests and sacrificed at respectively 8, 16 or 24 weeks of age. Muscle histopathology and expression of genes involved in muscle pathology were analysed for several skeletal muscles, while miRNA levels were assessed in serum. In addition, for Sgcd−/− mice heart pathology was assessed. Muscle function showed a gradual decline in both Sgca−/− and Sgcd−/− mice. Respiratory function was also impaired at all examined timepoints. Already at 8 weeks of age, muscle pathology was prominent, and fibrotic, inflammatory and regenerative markers were elevated, which remained relatively constant with age. In addition, Sgca−/− mice showed signs of cardiomyopathy from 16 weeks of age onwards. These results indicate that Sgca−/− and Sgcd−/− are relevant disease models for LGMD2D and 2F.

Introduction

The limb girdle muscular dystrophies (LGMDs) comprise the most heterogeneous collection of muscular dystrophies with over 30 subtypes known. They are identified according to their genetic defects with autosomal dominantly and recessively inherited LGMDs sub-grouped as LGMD1 and LGMD2, respectively. LGMDs are characterised by a progressive weakness of proximal muscles of the hip and shoulder girdles [1]. Sarcoglycanopathies comprise four subtypes, LGMD2C, -D, -E and -F, which form the more common variants of LGMD. They are
caused by mutations in the genes coding for the muscle-specific transmembrane sarcoglycan proteins α-, β-, γ-, and δ-sarcoglycan [2]. Sarcoglycans are crucial components of the dystrophin-glycoprotein complex that physically connects the intracellular cytoskeleton to the extracellular matrix. The loss of this structural linkage, for instance due to mutations in one of the sarcoglycan genes, makes muscle fibres more susceptible to damage during muscle contractions [3–6]. Although causative gene mutations are well known, there is currently no specific therapy available for sarcoglycanopathies [7].

Animal models for α- and δ-sarcoglycanopathies [B6.129S6-Sgca<sup>tm2Kcam</sup>/J (Sgca<sup>−/−</sup>; LGMD2D) and B6.129-Sgcd<sup>tm1Mcn</sup>/J (Sgcd<sup>−/−</sup>; LGMD2F) mice, respectively] have been generated mimicking many features of the human disease [8, 9]. We previously performed a comprehensive longitudinal natural disease history study in which we assessed muscle function in Sgca<sup>−/−</sup> and Sgcd<sup>−/−</sup> mice for 30 weeks (from 4 to 34 weeks of age) on a bi-weekly basis [10]. Although this study was instrumental for setting up standardized pre-clinical trials in LGMD2D and 2F mice, direct comparisons between muscle function and pathology at younger ages could not be made. Therefore, we here present a cross-sectional study in Sgca<sup>−/−</sup> and Sgcd<sup>−/−</sup> mice in which we correlate muscle function and pathology at 8, 16 and 24 weeks of age. This study provides a comprehensive insight in the age-related development of pathology in Sgca<sup>−/−</sup> and Sgcd<sup>−/−</sup> mice, which could facilitate their use in future pre-clinical drug trials.

**Materials and methods**

**Animals**

All experiments were approved by the Animal Experiment Committee (Dierexperimentencommissie) of the Leiden University Medical Center (protocol #13211) and executed following EU-guidelines. The Sgca<sup>−/−</sup> (B6.129S6-Sgca<sup>tm2Kcam</sup>/J; α-sarcoglycan-deficient) mice [11] were kindly provided by Queensta Millet, University College London and the Sgcd<sup>−/−</sup> (B6.129-Sgcd<sup>tm1Mcn</sup>/J; δ-sarcoglycan-deficient) mice [12] were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Males were used for all experiments. Mice were bred in the Experimental Animal Facility of the Leiden University Medical Center. They were kept in ventilated cages at 20.5˚C with 12 h of light/dark cycles and had ad libitum access to standard RM3 chow (SDS, Essex, UK) and water. Care was taken to limit the burden and distress for the animals as much as possible.

Twice monthly, Sgca<sup>−/−</sup>, Sgcd<sup>−/−</sup> and C57BL/6J wild type male mice (n = 18 per genotype) were subjected to the four limb grip strength test and two and four limb hanging tests on consecutive days, from the age of 4 weeks to either 8, 16 or 24 weeks. At these ages, six males per genotype were sacrificed by cervical dislocation and muscles were dissected for analysis (see below) to allow direct comparisons between muscle function and pathology, while the remaining mice continued this functional test regime. The functional tests conducted were suitable and sensitive tests for muscle function in LGMD mice, based on our previous longitudinal study [10]. Standardized operating procedures from the TREAT-NMD network for mdx mice were implemented wherever possible [13].

**Four limb grip strength test**

A grid attached to an isometric force transducer (Columbus Instruments, USA) was used to measure peak force of the fore and hind limbs. Hereto, the mouse was suspended, handled by the tail, above the grid which it naturally grasped with its four paws. The maximal force generated by the mouse to the grid when pulled away by the experimenter was recorded by the force transducer. This was repeated three times in a row after which the mouse got a short rest. The
mouse was subjected to five series of three pulls, each followed by a resting period (15 pulls in total). The three highest values of the 15 pulls were averaged and normalized to body weight.

**Two limb hanging test**

The mouse was suspended above a metal wire which was secured above a cage with bedding and released after it had grasped the wire with its fore limbs. The hanging time until fall was recorded. The mouse was allowed to use all four limbs and the tail during hanging if capable to do so. The test ended after a hanging time of 600 s was achieved or otherwise after three sessions. The maximum hanging time was utilized for analysis.

**Four limb hanging test**

The mouse was placed on a grid, which was then turned upside down above a cage filled with bedding. The test ended when a hanging time of 600 s was achieved or, upon earlier fall, after three sessions. The maximum hanging time was utilised for analysis.

**Respiratory function analysis**

Mice underwent respiratory function analysis with the whole-body plethysmograph (RM-80; Columbus Instruments, Columbus, OH, USA) [14] at the age of 8, 16 and 24 weeks. After 30 s acclimatization, the respiration signal was recorded for 120 s. The signal was digitized using a Minidigi digitizer and Axoscope 10 software (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) and analysed with the event detection feature of the Clampfit 10 program (Axon Instruments/Molecular Devices). This non-invasive monitoring system allowed for measurement of respiration rate and depth. The respiration amplitude was normalized to body weight.

**Muscle histology and morphology**

Directly after sacrifice, the gastrocnemius, quadriceps, triceps, diaphragm and heart were isolated and snap frozen in liquid nitrogen cooled isopentane. Muscles were embedded in OCT compound and sections (8 μm thickness) were cut with the cryotome (Leica CM3050 S Research Cryostat; Amsterdam, the Netherlands) while intermediate sections were collected for RNA isolation. For all microscopical analyses pictures were taken from the entire cross-sectional area of a section originating from the middle part of the muscle at a ten times magnification with a BZ-X700 microscope (Keyence, Japan) and stitched with a BZ-X700 analyser version 1.3.0.3 (Keyence). Adobe Photoshop CC 2018 (Adobe Systems Corporation, San Jose, CA, United States) was used for background correction and ImageJ software (NIH) for analysis.

To assess overall pathology, slides were fixed in ice-cold acetone (Avantor; Arnhem, the Netherlands) for 5 min, stained with hematoxylin and eosin (H&E, Sigma-Aldrich, Zwijndrecht, the Netherlands) and mounted in Pertex mounting medium (VWR International B.V; Amsterdam, the Netherlands), according to conventional methods. Images were analysed by three to four examiners and the median of their assessments was used for calculations. Here, the amount of healthy versus unhealthy tissue (consisting of necrosis, fibrosis, inflammation and newly regenerated fibres) was assessed.

Sections were stained with Alizarin Red to quantify calcification area in muscle. Hereto, sections were fixed in ice-cold acetone (Avantor) for 10 min and exposed to Alizarin Red staining solution (Sigma-Aldrich) for 1 min. Then, sections were washed in acetone (Avantor) for 30 s and in 1:1 acetone/xylene for 15 s and finally incubated in xylene for 1.5 h and mounted
in Pertex (VWR International B.V). The percentage of calcification was determined by two to three examiners by dividing Alizarin Red positive areas by the total area and the median of their assessments was used for final analysis.

For fibre size analyses, muscle sections were fixed with ice cold acetone (Avantor) and stained with a laminin primary antibody (ab11575, dilution 1:100 Abcam, USA) overnight at 4˚C and with a goat-anti-rabbit Alexa 594 secondary antibody (A11012, dilution 1:1000, Life Technologies) for 1 h at room temperature. Sections were mounted with mounting medium containing DAPI (ProLong Antifade Reagents; Life Technologies). Per section five randomly chosen microscopic views were analysed resulting in an average total number of 1500-4000 fibres analysed per muscle. Fibres with an area $<100 \mu m^2$ or $>10000 \mu m^2$ were excluded from analysis.

Levels of collagen were quantified by Sirius Red staining. Sections were fixed in 4% paraformaldehyde for 10 min and 100% ethanol for 5 min. Thereafter, they were air dried for 30 min and rinsed in deionized water. Sections were stained with Sirius Red solution (Direct Red 80; Sigma-Aldrich) for 45 min, followed by washing with 0.5% acetic acid water for 5 min and rinsed in deionized water. Stepwise dehydration in ethanol (80%--90%--100%) was performed and after incubation in xylene for two times 5 min, sections were mounted in Pertex (VWR International B.V). The three to four examiners assessed the percentage of fibrosis by dividing the Sirius Red positive areas by the total area. When large variations in fibrosis levels occurred for a sample between evaluators, these samples were discussed and a compromise was achieved. The median of their results was used for analysis.

The percentage of fibrosis was measured for the heart. Hereto, sections were stained with collagen type I primary antibody (1310-01 Southern Biotech, dilution 1:100, Birmingham, USA) overnight at 4˚C and with a donkey-anti-goat Alexa 488 secondary antibody (A11001, dilution 1:1000, Life Technologies) for 1 h at room temperature and mounted with mounting medium containing DAPI (ProLong Antifade Reagents; Life Technologies). Quantification of the collagen type I positive area and the total tissue area was performed by two examiners and the average of their acquired percentage of fibrosis was used for further analyses.

### RNA isolation and qPCR

Muscle sections were collected in 1.4 mm Zirconium Beads prefilled tubes (OPS Diagnostics, Lebanon, USA) and disrupted in TRIzol isolation reagent (GCBiotech, Alphen aan den Rijn, the Netherlands) using a MagNA Lyser (Roche Diagnostics). Total RNA was isolated and cleaned up by applying a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instruction. cDNA was synthesized from 0.3-0.5 μg of total RNA (depending on the muscle type but kept constant between comparisons of different genotypes and ages of the same muscle) using random N6 primers (Thermo fisher scientific) and BioScript enzyme (GCBiotech, Alphen aan den Rijn, the Netherlands) according to the manufacturer’s instructions. Gene expression levels were determined for cluster of differentiation 68 (Cd68), collagen type Iα1 (Col1a1), connective tissue growth factor (Ctgf), lectin, galactoside binding soluble 3 (Lgals3), lysyl oxidase (Lox), latent transforming growth factor beta binding protein 4 (Ltbp4), myosin heavy chain 3 (Myh3), myogenin (MyoG), NADPH oxidase 2 (Nox2), natriuretic peptide type A (Nppa), platelet derived growth factor receptor alpha polypeptide (Pdgfra), peroxisome proliferator-activated receptor γ (Pparγ), sarcoplasmic reticulum Ca$^{2+}$ ATPase2α (Serca2α), signal transducer and activator of transcription 3 (Stat3) and vascular endothelial growth factor (Vegf) in triplo by quantitative PCR, using SensiMix reagents (GCBiotech) and the LightCycler 480 (Roche), using a program consisting of 45 cycles of 95˚C (10 s), 58˚C (30 s) and 72˚C (20 s). The LinReg qPCR method was used to analyse gene
expression levels and values were normalized utilizing the housekeeping gene Hmbs. Primer sequences and gene functions are provided in S1 Table.

**miRNA analysis.** Mice were anaesthetized with isoflurane and blood was collected in an Eppendorf tube after orbital bleeding, after which mice were sacrificed by cervical dislocation. Clotting was allowed for 10 min at room temperature, and samples were subsequently stored on ice. Serum was obtained after 10 min spinning at 13,000 rcf and stored until further processing at -80°C. Serum samples of 8- and 16-weeks-old mice were used for analysis. Due to limited availability of serum from 24-weeks-old mice, serum samples of 34 weeks old C57BL/6j wild type, Sgcα/− and Sgcd/− males (n = 5–6 per group) of our previous natural history study [10] were included in further analyses. The set-up of this study was comparable to the previous one and samples had been obtained in the same manner. Total RNA from 100 μL serum was isolated in 500 μL TRIsure (Bioline, London, United Kingdom). Hereeto, 3 μL of three synthetic *Caenorhabditis elegans* miRNAs (cel-miR-39-3p, cel-miR-54-3p and cel-miR-238-3p) (Eurogentec, Maastricht, the Netherlands) with a concentration of 5 nM was added. cDNA synthesis was performed using 47.25 ng of total RNA. First, microRNAs (miRNAs) were polyA tailed using poly(A) polymerase (ABMgood, Richmond, BC, Canada) and oligo(dT) adapter (GARTCGGACACCAGTTACGATGCCAGGGCTGACTTCTTAGATTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
gastrocnemius, quadriceps and triceps and <500 μm² for diaphragm. A two-way ANOVA test was subsequently used between genotypes and age groups, followed by Tukey’s post hoc correction for multiple comparisons. Statistical significance was set at $p < 0.05$. All results of histological, gene expression and miRNA analyses are provided in S3 Table.

**Results**

**Muscle function**

*In vivo* muscle function of $Sgca^{-/-}$ and $Sgcd^{-/-}$ mice was assessed on a bi-weekly basis for a period of either 4, 12 or 20 weeks, starting at the age of four weeks. Functional test types were chosen based on previous experiments [10]. Functionality was compared to C57BL/6J wild type mice and only males were included as their muscle performance is more impaired than that of females in the $Sgcd^{-/-}$ strain [10]. Body weight did not differ between the genotypes (Fig 1A). No difference in muscle strength was observed between the genotypes as assessed by the four limb grip strength test (Fig 1B). To examine muscle function and fatigability two and four limb hanging tests were performed (Fig 1C and 1D). In both tests wild type mice greatly outperformed $Sgca^{-/-}$ and $Sgcd^{-/-}$ mice ($p < 0.01$), of which the muscle function declined with age. No differences were observed between $Sgca^{-/-}$ and $Sgcd^{-/-}$ mice.

Since respiratory dysfunction has been observed in LGMD patients and animal models [10, 17], whole-body plethysmography was performed. Respiratory amplitude, normalised to body weight, was lower in $Sgca^{-/-}$ and/or $Sgcd^{-/-}$ mice versus wild type mice at 8 and 16 weeks of age, but not at 24 weeks (Fig 1E). Respiratory rate was decreased in both LGMD models compared to wild type mice at all ages, and differed between the $Sgca^{-/-}$ and $Sgcd^{-/-}$ mice at 8 and 16, but not at 24 weeks of age (Fig 1F). Respiratory function did not decline with age in any of the genotypes.

**Muscle quality**

Overall muscle pathology (consisting of necrosis, fibrosis, inflammation and newly regenerated fibres) was assessed by H&E staining for the gastrocnemius, quadriceps, triceps and diaphragm (Fig 2 and S1 Fig). Already at 8 weeks of age, muscle quality was compromised in both $Sgca^{-/-}$ and $Sgcd^{-/-}$ compared to wild type mice for all examined muscles. Pathology did not differ between the $Sgca^{-/-}$ and $Sgcd^{-/-}$ models, and remained stable within the investigated age period for all muscles. The diaphragm was more severely affected than the limb muscles, which is in line with previous observations in sarcoglycanopathy and other dystrophic mouse models [10, 18, 19].

**Fibrosis and fatty infiltration**

During disease progression, degeneration of muscle fibres causes large infiltrates of inflammatory cells and eventually replacement of myofibrils by fibrotic and adipose tissue [11, 20]. To examine fibrosis, muscle sections were stained with Sirius Red (Fig 3A). Already from 8 weeks of age onwards, large areas of fibrosis were seen in all analysed muscles of both LGMD strains. Fibrotic lesions increased in abundance during ageing for some, but not all, muscles. In correspondence with the H&E staining quantification, the diaphragm contained the largest proportion of fibrosis.

Furthermore, gene expression levels of markers for fibrosis were measured (*i.e. Col1a1, Lox, Pdgfra, Ctgf and Ltbp4*). Out of these genes, the expression of *Col1a1* (Fig 3B), *Lox* (Fig 3C) and *Pdgfra* (Fig 3D) was most drastically upregulated in $Sgca^{-/-}$ and $Sgcd^{-/-}$ mice, already from early age onwards. For *Col1a1*, a small decrease with age was seen in the gastrocnemius
Fig 1. Functional performance and respiratory function of wild type, Sgca−/− and Sgcd−− males. (A) Body weight. (B) Four limb grip strength normalised for body weight. (C) Maximal two limb hanging times. (D) Maximal four limb hanging times. (E) Respiratory amplitude corrected for body weight. (F) Respiratory rate. N = 6–18 male mice per group. Values are presented as mean ± SD. *p<0.05, **p<0.01.

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and triceps (Fig 3B). *Pdgfra* levels were upregulated in all muscles except for the diaphragm of *Sgca*<sup>−/−</sup> and *Sgcd*<sup>−/−</sup> mice. Furthermore, in the gastrocnemius of both LGMD models a small decrease over time was seen, whereas levels were increased in the triceps (Fig 3D). More moderate changes were observed for *Ctgf* (Fig 3E) and *Ltbp4* expression (Fig 3F). *Ctgf* expression was increased in *Sgcd*<sup>−/−</sup>, but not in *Sgca*<sup>−/−</sup> males. *Ltbp4* expression solely increased at 24 months, mainly in *Sgcd*<sup>−/−</sup> males (Fig 3F).
Fig 3. Fibrosis and fat formation in Sgca<sup>−/−</sup> and Sgcd<sup>−/−</sup> males. (A) Quantification of fibrosis by Sirius Red staining. (B-F) Gene expression of fibrotic markers Col1a1 (B), Lox (C), Pdgfra (D), Ctgf (E) and Ltbp4 (F). (G) Gene expression of Pparγ, a marker for fatty infiltration. N = 5-6 male mice per group. *p<0.05 vs age-matched wild type mice, #p<0.05 over time (same genotype).

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Expression of Pparγ, a regulator of adipocyte differentiation, was assessed as a marker for fatty infiltration. Increased Pparγ expression was seen in both LGMD mouse strains, most prominently in the gastrocnemius. In the diaphragm, however, Pparγ expression did not differ between wild type and dystrophic mice (Fig 3G).

Regeneration

Cycles of degeneration and regeneration are a hallmark of muscular dystrophies. Fibre size distribution was determined utilising a laminin staining to segment individual fibres (Fig 4A–4D). A shift towards smaller fibre sizes, an indicator of regeneration, was seen in all examined muscles of both LGMD models. At all ages, most fibres were smaller than 1000 μm² for gastrocnemius, quadriceps and triceps for Sgca−/− and Sgcd−/− mice, whereas in wild type mice the majority was 1000-3000 μm² (Fig 4A–4C). In the diaphragm, smaller fibre sizes were observed in general, also for wild type mice, compared to the other muscles. Even though a shift towards smaller fibres was seen in Sgca−/− and Sgcd−/− mice, this was less prominent than for other muscles (Fig 4D). No large changes over time were observed in the examined muscles, neither did fibre size differ between the two LGMD models.

Expression levels of several regeneration markers (Myh3, MyoG, Nox2 and Stat3) were assessed (Fig 4E–4G). The expression of most genes was upregulated in the LGMD models from 8 weeks of age onwards and few changes over time were observed. Myh3, expressed by recently regenerated fibres, was nearly undetectable in wild type mice. In Sgca−/− and Sgcd−/− mice, however, significantly higher levels were found in all analysed muscles at most time points (Fig 4E). MyoG, a marker of early regeneration, was also upregulated in LGMD mice (Fig 4F). Nox2, a marker of regeneration and inflammation, was upregulated in all muscles of Sgca−/− and Sgcd−/− mice regardless of their age (Fig 4G). Less abundant changes were seen for Stat3. Expression was only statistically significantly increased in triceps of both dystrophic models at all time points (except for Sgca−/− mice at 8 weeks of age), and in gastrocnemius and quadriceps of 24 weeks old mice Sgca−/− and/or Sgcd−/− mice (Fig 4H). For Sgca−/− mice also a small increase over time was observed in quadriceps.

Inflammation and calcification

Another hallmark of dystrophic muscle is the infiltration of inflammatory cells. To measure the extent of inflammation, expression levels of Cd68 and Lgals3 were determined (Fig 5A and 5B). Expression of both markers was upregulated in all examined muscles of the Sgca−/− and Sgcd−/− mice at almost all timepoints. Similarly to findings for fibrosis and regeneration, no differences during ageing were observed.

To assess whether calcification is involved in the pathology of Sgca−/− and Sgcd−/− mice, muscle sections were stained with Alizarin Red. Whereas no calcified fibres were observed in wild type mice, skeletal muscles of both sarcoglycanopathy mouse models contained small groups of calcified fibres, regardless of the animal’s age (Fig 5C). The extent differed between individuals.

miRNA

miRNAs regulate mRNA expression by post-transcriptional cleavage or repression [21, 22]. They show tissue-specific expression patterns and play a role in several pathophysiological conditions, among which muscular dystrophies [23, 24]. Upon muscle damage miRNAs can leak into the bloodstream. Therefore, we looked in serum at the levels of several miRNAs, involved in myogenic proliferation and/or differentiation [23, 25–29] (for more information on specific roles see S2 Table). Some of these are specifically expressed in muscle (myomiRs)
Fig 4. Regeneration in Sgca−/− and Sgcd−/− males. (A-D) Muscle fibre area distribution measured by staining with laminin. (E-H) Gene expression of Myh3 (E). Since Myh3 was not detectable in wild type mice, values were put at zero. MyoG (F), Nox2 (G) and Stat3 (H). N = 5-6 male mice per group. *p<0.05 vs age-matched wild type mice, #p<0.05 over time (same genotype).

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With limited sample available for the 24 weeks old mice, we used serum of 34 weeks old \textit{Sgca}\(^{-/-}\), \textit{Sgcd}\(^{-/-}\) and C57BL/6J males instead, collected in our previous natural history study [10]. Mainly in young \textit{Sgca}\(^{-/-}\) and \textit{Sgcd}\(^{-/-}\) mice, increased levels of miR-1a and miR-206, mi-RNAs which promote myogenic differentiation and are abundant in muscle, were observed (Fig 6A and 6B). However, no differences were observed for miR-30e (Fig 6C) and miR-148a (S2C Fig). Increased levels of miR-133a (S2D Fig) and miR-133b (Fig 6D) are indicative for active myoblast proliferation in \textit{Sgca}\(^{-/-}\) and \textit{Sgcd}\(^{-/-}\) males. A small decrease in expression during ageing was observed in all genotypes, although this was only significant in \textit{Sgcd}\(^{-/-}\) mice. miR-434 has been shown to inhibit apoptosis and is downregulated in mice during ageing [30]. No differences were observed between the LGMD models and the wild types (S2E Fig).

**Cardiac involvement**

Cardiomyopathy has been observed in \textit{Sgcd}\(^{-/-}\) mice [9, 10] and LGMD2F patients [8, 31], but not in \textit{Sgca}\(^{-/-}\) mice [10]. Therefore, heart pathology was only assessed in \textit{Sgcd}\(^{-/-}\) mice. To assess cardiac hypertrophy, the heart to body weight ratio was determined. No differences with wild type mice were observed at any age (S3A Fig). Furthermore, the amount of collagen (fibrotic tissue) was analysed (Fig 7A). Whereas in young \textit{Sgcd}\(^{-/-}\) mice no cardiac fibrosis was observed yet, from the age of 16 weeks onwards some fibrotic areas were present in the dystrophic mice. A modest increase in expression of fibrotic genes (\textit{Col1a1} and \textit{Ctgf}) was also observed, mainly for \textit{Ctgf} (Fig 7B and 7C). The increased fibrosis in the \textit{Sgcd}\(^{-/-}\) hearts was not accompanied by an altered expression of genes involved in cardiac function (\textit{Serca2a} [32, 33], \textit{Nppa} [34] and \textit{Vegf} [35]) (S3B–S3D Fig).
Fig 6. miRNA serum levels. (A-D) Relative levels of miR-1a (A), miR-206 (B), miR-30e (C) and miR-133b (D) in 8, 16 and 34 weeks old mice. N = 4-6 male mice per group. For miR-30e, the level of 16-weeks-old wild type mice is based on n = 3. Levels of 8-weeks-old wild type mice were put at 1. *p<0.05 vs age-matched wild type mice, #p<0.05 over time (same genotype).

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Fig 7. Heart pathology in Sgcd<sup>-/-</sup> males. (A) Collagen type I staining of fibrotic area. Gene expression of Col1a1 (B) and Ctgf (C). N = 6 male mice per group. *p<0.05 vs age-matched wild type mice, #p<0.05 over time (same genotype).

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Discussion

At the moment, specific therapies for the sarcoglycanopathies LGMD2D and 2F are lacking and patients are treated according to the care guidelines [36, 37]. For LGMD2D clinical research into gene therapy is ongoing, but for LGMD2F therapeutic development is still only in pre-clinical stages [38, 39]. Animal models are important tools for understanding disease mechanisms and pre-clinical research on potential therapies. Although description of their natural course of the disease is vital for these studies, full characterization of the Sgca\(^{-/-}\) and Sgcd\(^{-/-}\) mice is lacking. In a previous study we assessed the disease pathology in adult mice (at 34 weeks of age), which showed a severe phenotype [10]. To determine if disease features are already present at younger ages and how they develop over time, we here investigated several pathological hallmarks in 8-, 16- and 24-weeks-old mice.

In vivo muscle function was already impaired in the youngest mice investigated (8 weeks of age) and showed a rapid decline with age. These results are in line with those obtained during the previous natural history study [10]. Especially in the hanging tests large differences with wild type mice were observed, similar to those seen in mouse models for Duchenne muscular dystrophy (DMD) [13, 40]. Respiratory dysfunction is common in sarcoglycanopathies [1, 17, 31] and was indeed already seen in both models at young age. However, in contrast to human patients, no further decline was seen during ageing. While in the previous study respiratory amplitude of 15-weeks-old Sgca\(^{-/-}\) and Sgcd\(^{-/-}\) mice only showed a small tendency towards decline [10], in the current study this decrease was statistically significant at 16 weeks of age. Results obtained for the respiratory rate do correspond between both studies.

Histological analyses showed that all examined muscles, especially the diaphragm, were severely affected in both LGMD models. Fibrotic, inflammatory and necrotic tissues were prominent. Furthermore, muscles were characterized by small calibre fibres which contained centrally located nuclei, indicative of muscle regeneration in the LGMD models at all ages. Especially in skeletal muscles, occasional groups of calcified fibres were present in both LGMD models. This was however not as extensive as that seen in the D2-mdx (a mouse model for DMD) and β-sarcoglycan deficient mice [41–43]. Already at eight weeks of age muscles were severely affected and remained relatively stable over time. It is likely that disease pathology starts at early age. It would therefore be interesting to investigate neonatal mice in future studies.

Several secondary signalling pathways are also disturbed due to the absence of sarcoglycans. These are, amongst others, pathways involved in fibrosis, adiposis, inflammation and regeneration. We here showed that several genes involved were upregulated in both LGMD models. Most markers already showed differential expression at eight weeks of age and remained relatively stable over time. In addition to gene expression, also miRNAs are valuable biomarkers for disease mechanisms. Especially myomiRs involved in myoblast proliferation and differentiation (e.g. by affecting MyoG and/or MyoD signalling), such as miR-1a, miR-133a/b and miR-206 [23, 28], were increased in the bloodstream of Sgca\(^{-/-}\) and Sgcd\(^{-/-}\) mice at most ages, suggestive of ongoing regeneration and damage. This confirms an early study showing an increase of these miRNAs in Sgca\(^{-/-}\) mice [44]. To our knowledge miRNA serum levels have not been examined specifically in LGMD2D patients or LGMD2F patients/mouse models. Similar changes, however, have been observed in other muscular dystrophies, such as other types of LGMD (LGMD2A, 2B and 2C) and DMD [24, 44–46]. In one study on the miR profile of LGMD2A/B patients, contrasting effects were found (a decrease in muscle miR levels rather than an increase) [47]. It could be that the rate of regeneration differs between LGMD types 2A/B and types 2D/F. Another explanation may be that in that study muscle tissue was used, whereas in our, and most other, studies, serum samples were used. Increased miR serum levels can only been observed if these miRs also leak out of the muscle tissue into the bloodstream.
Therefore, it would be interesting to investigate miR expression levels in muscle tissue of Sgca⁺/⁻ and Sgcd⁺/⁻ mice.

Cardiac involvement is common in LGMD2F patients, but not in LGMD2D patients [17, 48]. This is because δ-sarcoglycan, but not α-sarcoglycan, is also expressed in cardiomyocytes and arterial smooth muscle cells. Its absence leads to myofiber damage and causes disturbances in the vascular function, leading to cardiomyopathy [9, 49]. We indeed confirmed previously that only Sgcd⁺/⁻ mice suffer from cardiomyopathy [10], and here showed that fibrosis in the heart could be detected from 16 weeks of age onwards. This was accompanied by elevated expression of fibrotic markers in the heart, but not by altered expression of genes related to heart function. More in depth analysis of cardiac function of Sgcd⁺/⁻ mice would be of interest. Future studies should focus in greater detail on the involvement of cardiomyopathy in these mice.

Overall the results indicate that Sgca⁺/⁻ and Sgcd⁺/⁻ mice resemble the muscular dystrophy features observed in patients, with an early onset of pathology [2, 36]. This allows utilization of these models for the testing of therapeutic interventions from an early age onwards. Especially examination of effects on the diaphragm is important, since this is the most severely affected muscle in both models and most likely also in patients. Further studies are needed to establish at which age pathology is starting in these strains. An important difference between LGMD2D and 2F patients and the mouse models is that the disease progression is limited in mice. While muscle function steadily declined, muscle pathology did not drastically increase in severity with age. This could have several reasons. Firstly, mice are known for their relatively good muscle regeneration capacity, which was also observed in these mice. In mdx mice cycles of de- and regeneration occur between 3 to 8 weeks of age, whereupon stabilisation of the disease is seen due to the regeneration and low levels of necrosis [50, 51]. This might also be the case for Sgca⁺/⁻ and Sgcd⁺/⁻ mice. Secondly, the relative length of the growth and maturation period is much shorter in mice compared to humans. Thirdly, the load on the muscles is reduced in mice due to their small body size and, since mice walk on four limbs and humans on two, different muscles are stressed [52].

In conclusion, in addition to our previous study of 34-weeks-old mice, this cross-sectional study further underscores that Sgca⁺/⁻ and Sgcd⁺/⁻ mice are representative models for LGMD2D and LGMD2F, respectively. Therefore they are useful for pre-clinical testing of therapeutic interventions.

Supporting information

S1 Fig. Histological analysis of muscle quality. Representative images (20x magnification) of H&E staining of the gastrocnemius of each genotype and age group. Scale bars represent 100 μm. (TIF)

S2 Fig. miRNA serum levels. (A-E) Levels of miR-23a (A), miR-451a (B), miR-148a (C), miR-133a (D) and miR-434 (E) in 8, 16 and 34 weeks old males. N = 3-6 male mice per group, except for miR-133a in 8-weeks old Sgca⁺/⁻ mice (n = 2). *p<0.05 vs age-matched wild type mice, #p<0.05 over time (same genotype). (TIF)

S3 Fig. Heart pathology of Sgcd⁺/⁻ mice. (A) Heart to body weight ratio. (B-D) Expression of genes related to heart function Serca2α (B), Nppa (C) and Vegf (D). N = 6 male mice per group. (TIF)
S1 Table. Primer sequences and functions of genes used for gene expression analysis.

S2 Table. Primer sequences used for miRNA analysis.

S3 Table. Statistical analysis of histology, gene expression and serum miRNA levels.
NA = not applicable; ND = not determined; ns = not significant. p-values after correcting for multiple testing.

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