Potential therapies and biomarkers for LAMA2-CMD

Potential therapies and biomarkers for LAMA2-CMD:

Does the microRNA hype deliver?

Bernardo Moreira Soares Oliveira

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalk salen, BMC A1, Lund on 2018-09-01 at10:00.

Faculty opponent
Dr. Kanneboyina Nagaraju

Organization: LUND UNIVERSITY	Document name: Doctoral thesis
	Date of issue: 2018-08-10
Author(s): Bernardo Moreira Soares Oliveira	Sponsoring organization: CAPES/CNPq

Title and subtitle: Potential therapies and biomarkers for LAMA2-CMD: does the microRNA hype deliver?

Abstract

Laminin α2 chain-deficient muscular dystrophy, or LAMA2-CMD, is a very severe disease caused by mutations in the LAMA2 gene. Skeletal muscle is the most affected tissue, with patients presenting symptoms such as hypotonia at birth, joint contractures and progressive muscle wasting. Changes in the central nervous system include white matter abnormalities, delayed motor milestones and compromised action potential propagation. To date, there is no cure and most therapeutic interventions aim at alleviating secondary complications. In the last two decades microRNAs (miRNAs) have been explored as biomarkers or therapeutic targets in various diseases. In this thesis we have profiled miRNAs in quadriceps muscle of the dy3K/dy3K mouse model of LAMA2-CMD as an initial screening. We then investigated the effects of genetically removing a pro-fibrotic miRNA, i.e. miR-21, in two mouse models of LAMA2-CMD. We showed that the single deletion of this miRNA is not enough to reduce fibrosis and improve muscle phenotype or function in LAMA2-CMD mouse models. We further explored miRNAs as non-invasive biomarkers of disease progression. Urine miRNAs were profiled at 3 time points representing no symptoms, initial symptoms and severe disease (3, 4 and 6 weeks of age). We found distinct panels of differentially expressed miRNAs at these time points, suggesting that miRNAs can be used as biomarkers for LAMA2-CMD progression. Finally, we explored metabolism as a therapeutic target in a metformin intervention study. We found that metformin treatment improved grip strength in treated dy2J/dy2J mice, despite unaffected muscle weights. Energy efficiency was improved in treated dy2J/dy2J females, which resulted in improved weight gain. Central nucleation decreased in treated dy2J/dy2J mice, which suggests reduced muscle damage. We found a significant reduction of small fibres in dy2J/dy2J females with metformin. White adipose tissue weight increased in treated dy2J/dy2J mice; in contrast, brown adipose tissue weight was reduced in treated dy2J/dy2J males.

Key words: laminin alpha 2, muscular dystrophy, microRNA, fibrosis, metabolism, skeletal muscle

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-7619-666-3
Recipient's notes	Number of pages	Price
	Security classification	

Signature Date 2018-07-30

Potential therapies and biomarkers for LAMA2-CMD:

Does the microRNA hype deliver?

Bernardo Moreira Soares Oliveira

LUND
UNIVERSITY

Coverphoto by Bernardo Moreira Soares Oliveira

Faculty of Medicine
Department of experimental medical science

ISBN 978-91-7619-666-3
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2018

To my kin and my friend Masa Ruotniemi (RIP)

Table of contents

Acknowledgements

First and foremost, my kin, for the never-ending support and love. My wife, my partner in crime, there are no words that could thank you. We're finishing another chapter in our 'Book of Adventures' and will soon start another one. With you by my side the sky is the limit. Towards the stars!

Cibely, for introducing me to the group and opening the doors. Thanks for all the supervision and advice! All the best to you and Joan Paul!

All of the Durbeej group, present and past, for the support and patience. It's been a great journey! In special, my mentors Madde and Johan for all the opportunities and help. Tusen tack!

Vahid and Vignesh for keeping the mood lively! All the best in the future.

List of figures

All images used in this thesis are under a Creative Commons licence.

Figure 1A – skeletal muscle macro-structure.
https://cnx.org/contents/bfiqsxdB@3/Skeletal-Muscle

Figures 1B and 2 – laminin and interacting proteins.
http://jcb.rupress.org/content/201/4/499

Figure 3 – miRNA biogenesis.
https://commons.wikimedia.org/wiki/File:MiRNA-biogenesis.jpg

Figure 4 – Next-generation sequencing methods.

https://www.slideshare.net/ueb52/introduction-to-next-generation-sequencing-v2

List of articles

I – Oliveira BMS, Holmberg J and Durbeej M. Profiling skeletal muscle microRNAs and their targets in the *dy^{3K}/dy^{3K}* mouse model of laminin α2 chain-deficient muscular dystrophy (manuscript).

II – Oliveira BMS, Durbeej M and Holmberg J. Absence of microRNA-21 does not reduce muscular dystrophy in mouse models of LAMA2-CMD. PLoS ONE. 2017, 12(8), e0181950.

III – Oliveira BMS, Gawlik KI, Durbeej M and Holmberg J. Exploratory profiling of urine microRNAs in the *dy^{2J}/dy^{2J}* mouse model of LAMA2-CMD: relation to disease progression (submitted).

IV – Fontes-Oliveira CC, Oliveira BMS, Körner Z, Harandi VM and Durbeej M. Effects of metformin on congenital muscular dystrophy type 1A disease progression in mice: a gender impact study (submitted).

Abstract

Laminin α2 chain-deficient muscular dystrophy, or LAMA2-CMD, is a very severe disease caused by mutations in the ***LAMA2*** gene. Skeletal muscle is the most affected tissue, with patients presenting symptoms such as hypotonia at birth, joint contractures and progressive muscle wasting. Changes in the central nervous system include white matter abnormalities, delayed motor milestones and compromised action potential propagation. To date, there is no cure and most therapeutic interventions aim at alleviating secondary complications. In the last two decades microRNAs (miRNAs) have been explored as biomarkers or therapeutic targets in various diseases. In this thesis we have profiled miRNAs in quadriceps muscle of the dy^{3K}/dy^{3K} mouse model of LAMA2-CMD as an initial screening. We then investigated the effects of genetically removing a pro-fibrotic miRNA, i.e. miR-21, in two mouse models of LAMA2-CMD. We showed that the single deletion of this miRNA is not enough to reduce fibrosis and improve muscle phenotype or function in LAMA2-CMD mouse models. We further explored miRNAs as non-invasive biomarkers of disease progression. Urine miRNAs were profiled at 3 time points representing no symptoms, initial symptoms and severe disease (3, 4 and 6 weeks of age). We found distinct panels of differentially expressed miRNAs at these time points, suggesting that miRNAs can be used as biomarkers for LAMA2-CMD progression. Finally, we explored metabolism as a therapeutic target in a metformin intervention study. We found that metformin treatment improved grip strength in treated dy^{2J}/dy^{2J} mice, despite unaffected muscle weights. Energy efficiency was improved in treated dy^{2J}/dy^{2J} females, which resulted in improved weight gain. Central nucleation decreased in treated dy^{2J}/dy^{2J} mice, which suggests reduced muscle damage. We found a significant reduction of small fibres in dy^{2J}/dy^{2J} females with metformin. White adipose tissue weight increased in treated dy^{2J}/dy^{2J} mice; in contrast, brown adipose tissue weight was reduced in treated dy^{2J}/dy^{2J} males.

Abbreviations

BMD – Becker muscular dystrophy

CNS – central nervous system

COPD – Chronic obstructive pulmonary disease

DMD – Duchenne muscular dystrophy

ECM – extracellular matrix

IGF-1 – insulin-like growth factor 1

LAMA2-CMD – laminin α2 chain-deficient muscular dystrophy

LGMD – limb-girdle muscular dystrophy

miR – microRNA (the mature form)

miRNA – microRNA

MRF – muscle regulatory factor

NMJ – neuromuscular junction

SC – satellite cell

TGF-β – transforming growth factor β

Background

Skeletal muscle

Skeletal muscle comprises about 40% of our body weight under normal conditions. It allows us to perform daily activities such as walking and lifting, and it's also responsible for vital functions such as eating, breathing and blood pumping. Skeletal muscle is the main energy consumer in our bodies and therefore plays an important role in metabolic homoeostasis.

Skeletal muscle mass is determined by the balance between muscle breakdown, or catabolism, and muscle build up, or anabolism. These processes are influenced by numerous environmental and physiological cues such as diet, exercise, stress and hormones, for example. These signals can tilt the balance in one direction or the other, resulting in net muscle gain or loss.

Over 70 million people suffer from muscle wasting disease, in one form or another, and this costs the health care industry over $500 billion annually. Yet, there are currently no treatments. These numbers are likely to increase as the elderly proportion increases and age-related muscle wasting (i.e. sarcopenia) becomes more prevalent. Moreover, muscle atrophy isn't an exclusive feature of myodegenerative diseases; it is also a secondary complication of diseases such as cancer cachexia [1], diabetes [2] and COPD [3], for example.

Not only treatments are lacking but also effective ways to assess them. In practice most clinicians resort to muscle biopsies to monitor disease progression, which is invasive, especially for children. They also measure creatine kinase (CK) concentration in blood, a muscle enzyme that leaks into circulation upon muscle damage; it is an indirect measure of muscle breakdown. Unfortunately, CK levels vary with factors such as sex, age, diet and stress, making it an unreliable marker [4]. There is thus a great need for better biomarkers for myopathies.

Myogenesis

The term myogenesis refers to skeletal muscle formation, especially during embryonic development. It is a complex process that depends on timely expression/repression of several genes. Generally speaking, the process involves a) the commitment of pluripotent cells to the myogenic lineage, b) proliferation of these cells followed by differentiation into myotubes and c) fusion of myotubes into myofibres [5; 6].

During the embryonic phase the cells that will give rise to skeletal muscle come from the (paraxial) mesoderm. These cells will form somites, which are parallel bundles of cells that run along the neural tube. The somites start subdividing into sclerotome (which will generate cartilage) and dermomyotome. The latter will be further divided into dermatome (skin) and myotome (muscle). The commitment of myotome cells to become myoblasts is regulated by transcription factors such as myogenic factor 5 (Myf5), myogenin and muscle regulatory factor 4 (MRF4), the main one being myogenic determination protein 1 (MyoD). These factors will initiate the myogenic programme in cells [5; 6].

Myoblasts will proliferate under the influence of MyoD and Myf5. When a sufficient number of cells is reached the myoblasts will exit the cell cycle and start to fuse into myotubes. Myotube formation occurs in two waves: the primary wave consists of embryonic myoblasts that will fuse to form mainly slow-contracting type I fibres; the second wave will have foetal myoblasts fuse to form (mostly) fast-contracting type II fibres. After birth, the number of skeletal muscle fibres is largely set. Therefore, adult skeletal muscle cannot increase fibre number (hyperplasia) but only regulate their size (hypertrophy or atrophy) [5; 6].

Regeneration

In post-natal muscle, especially adult muscle, the process of regeneration resembles that of (embryonic) myogenesis. Post-natal muscle retains stem-like cells that can differentiate into myotubes to aid muscle regeneration. These are called satellite cells (SCs) due to their peripheral position: they are located between the sarcolemma and the basement membrane, normally in a quiescent state. They proliferate in response to muscle damage and migrate to the injury site to fuse into myotubes, providing additional nuclei for transcriptional power [5; 6] .

Structure

Macroscopically, skeletal muscle is formed by fibre bundles surrounded by connective tissue. The latter will have different names depending on location: epimysium surrounds the whole muscle, perimysium covers fibre bundles and endomysium individual muscle fibres (figure 1A). Muscle fibres are the cellular unit of skeletal muscle. They're composed of myofibrils, which are contractile filaments made up mainly of actin and myosin. Other important myofibrillar proteins include titin and nebulin. Myofibrils are in fact a series of sarcomeres, the contractile unit of skeletal muscle. It is the cumulative shortening of sarcomeres that makes muscles contract [7; 8].

The sarcomere is divided into regions according to its interaction with polarised light (figure 1B). The boundaries of the sarcomeres can be seen under the microscope as narrow dense lines, the Z lines (from the German *zwischen*, between). A dense dark band can be observed in the middle of the sarcomere; it's the A band (from anisotropic). Its colour comes from a higher density of thick filaments such as myosin. The area between the A band in one sarcomere and the A band in the adjacent one is called I band (from isotropic). At the very centre of the A band is the M line (from the German *Mittelscheibe*, middle disk). The lighter region in the middle of A bands is the H zone (from the German *heller*, lighter), where thin and thick filaments do not overlap. The width of this region varies with muscle contraction [8] .

In order to contract skeletal muscle needs input from the central nervous system (CNS). Under voluntary contractions this command is generated in the motor cortex and transmitted down the spinal cord. In the anterior horn of the spinal cord the impulse is transmitted to a motor neuron and then passed to muscle cells. Each motor neuron, along with the fibres it innervates, is called a motor unit. The axon terminal and the sarcolemma region adjacent to it constitute the neuromuscular junction (NMJ). This is a specialised interface between neuron and muscle where the action potential is passed from the neuron to the muscle cell [8].

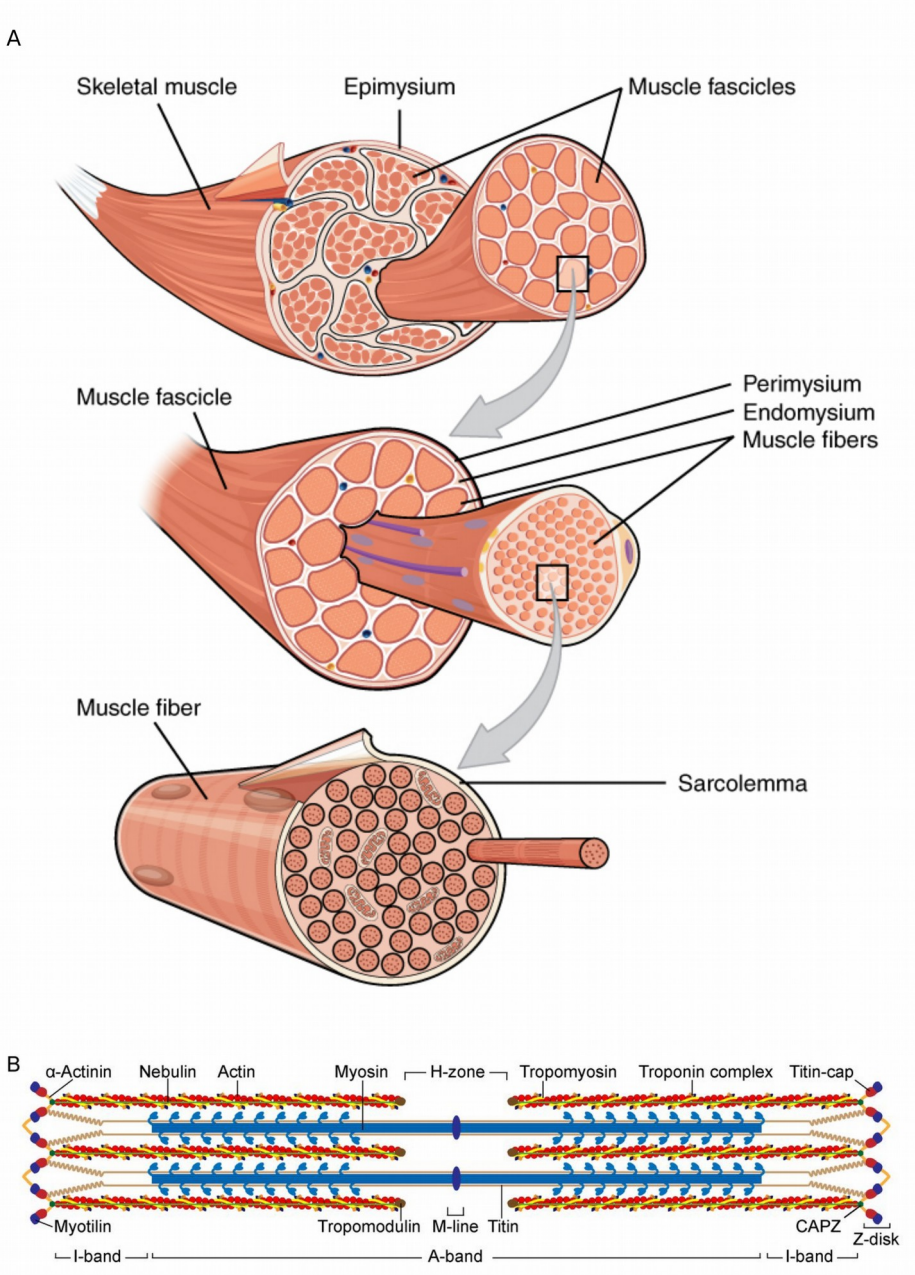

Figure 1.
Skeletal muscle hierarchical structure. A: macro-structure of muscle fibres and surrounding connective tissue. B: structure of the sarcomere, the contractile unit of skeletal muscle.

Extracellular matrix

The extracellular matrix (ECM) is a mesh-like network, composed mainly of polysaccharides and proteins, that provides structural and biochemical support to cells. It provides a scaffold to which cells can adhere and mature, as well as receptors and cytokines to modulate cellular activity. The ECM may store growth factors and cytokines in latent form, as well as proteases to process these into bioactive molecules, a prominent example being TGF-β [9] .

Polysaccharides constitute the ECM foundation where proteins will be inserted. They form coarse structures due to their stiffness and hydrophilicity. Their net negative charge attracts sodium ions that in turn attract water, keeping the cells and tissues hydrated. The main ECM polysaccharide is glycosaminoglycan (GAG) [7].

The most abundant ECM protein is collagen. In skeletal muscle it accounts for one to two percent of the tissue. It is synthesised and exported by fibroblasts as pro-collagen, and subsequently cleaved by ECM proteases. Collagen is composed of 3 tightly packed α chains in a helical structure. So far more than 20 α chains have been discovered, which in turn form approximately 20 collagen types [7; 9].

Basement membrane

The basement membrane (BM) is a specialised region of the ECM: it is a thin sheet that covers cells and separate them from, but also anchor to, the rest of the ECM. The BM is divided into (interior) basal lamina and (exterior) reticular lamina; the former is rich in non-fibrillar collagen, non-collagenous glycoproteins and proteoglycans, whilst the latter contains fibrillar collagen and proteoglycans [10]. The major components of BMs are collagen IV, laminins, heparan sulfate, fibronectin, nidogen and agrin [9; 11; 12] .

The BM has critical roles in mediating cell stability and integrity. Its structural role was described first and is thus better studied. The BM provides much of a muscle’s tensile strength and therefore prevents contraction-induced damage. Furthermore, even in the event of muscle damage the BM serves as a scaffold to support and direct SCs and promote proper muscle architecture [10]. The BM also covers other structures and tissues that

directly interact with skeletal muscle, such as myotendinous junctions and NMJs. For example, the basal lamina spans the cleft between muscle and nerve at the NMJ and keeps this critical structure in proper placement. At myotendinous junctions the basal lamina covers invaginations, increasing the surface area of muscle-tendon interaction and therefore aiding force transmission [7; 9].

Laminin

Laminins are heterotrimers formed by an α, a β and a γ subunit (or chain). Their names come from their composition: e.g. laminin-111 is composed of α1, β1 and γ1 chains. For the most part, their structure resembles a cross, with the β and γ chains protruding laterally and the α one upwards (figure 2). To date, over 15 laminin isoforms have been discovered.

Laminins are the major non-collagenous component of the basement membrane. In particular, laminin-211 is the most abundant isoform in skeletal muscle. Similarly to the ECM, laminins have structural (primary) and biochemical (secondary) roles. Much of the stiffness and tightness of the ECM is due to laminin polymerisation. Both N- (LN) and C-terminal (LG, globular) parts of the protein are crucial to its activity. The LN domain mediates self-assembly and polymerisation, whilst the LG domain binds to cell surface receptors such as integrin α7β1 and α-dystroglycan (figure 2). Thereby, laminins are linked to the intracellular cytoskeleton and transduce extracellular signals to the cytoplasm and hence modulate cellular activity. Laminins are involved in cell adhesion, migration and differentiation. The biological function of laminins is highly dependent on which receptor they interact with. Signalling through integrin α7β1 promotes cell growth and myofibre survival by activating the PI3K/Akt pathway. The lack of laminin α2 and the consequent reduction in Akt signalling stimulate processes involved in muscle atrophy such as apoptosis and the ubiquitin-proteasome system [11]. Less is known about laminin-211 signalling through α- and β-dystroglycan.

The laminin profile in muscle is time- and space-dependent. Some isoforms, such as laminin-111, are only expressed during embryonic stages. Others, are only expressed in specialised regions such as the NMJ (α4, α5 and β5) [9] .

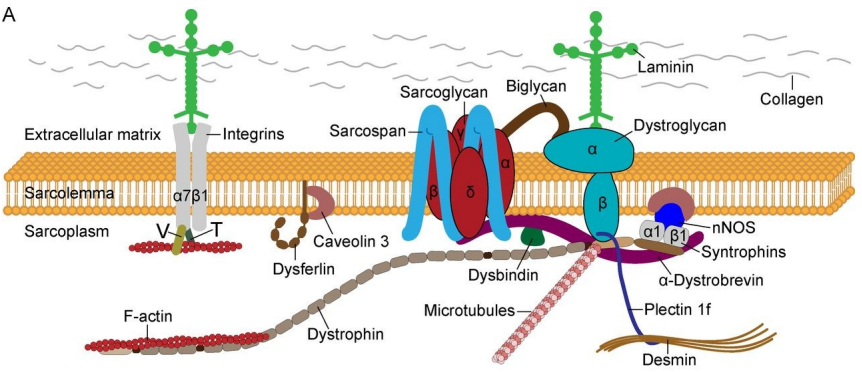

Fig 2.
Laminin and surrounding proteins. The main laminin-interacting proteins are integrins and dystroglycan.

Muscular dystrophies

Muscular dystrophies are a group of more than 50 genetic diseases that affect the neuromuscular system, resulting in progressive muscle loss. Other hallmark features include fibrosis and muscle inflammation. Many muscular dystrophies arise from mutations in genes encoding proteins of the dystrophin-glycoprotein complex (DGC). The DGC is a very large protein complex that sits on the muscle cell membrane (sarcolemma) and works to stabilise it and connect it to the ECM (figure 2). When this connection is faulty, muscle cells become prone to damage or detachment, which ultimately leads to muscle degeneration [13].

The most frequent type of the disease is Duchenne muscular dystrophy (DMD). It is an X-linked illness that affects mostly boys and causes loss of dystrophin, a protein involved in muscle membrane stability. Becker muscular dystrophy (BMD) is a milder variant of DMD due to lower levels of dystrophin or a truncated protein. Mutations in the laminin-interacting sarcoglycan complex lead to limb-girdle muscular dystrophies (LGMD) types 2C, 2D, 2E or 2F, depending on the particular isoform affected. LGMD is in

fact a group of very heterogeneous muscular dystrophies with the common feature that the proximal muscles of trunk and limbs are affected [13-15] .

Laminin α2 chain-deficient muscular dystrophy

Laminin α2 chain-deficient muscular dystrophy (LAMA2-CMD), also called merosin-deficient muscular dystrophy, or MDC1A, is a severe form of the disease caused by mutations in the *LAMA2* gene. Despite being a monogenic defect, the absence of laminin α2 chain leads to multiple complications at the tissue and cellular level. Its symptoms include substantial hypotonia at birth, muscle contractures, progressive muscle atrophy and fibrosis. The lack of laminin α2 chain affects not only skeletal muscle but also the central nervous system. Reported abnormalities include changes in white matter, defective myelination and slower action potential propagation. Thus, LAMA2-CMD, unlike most muscular dystrophies, affects the neuromuscular system as a whole [16].

The lack of proper attachment to the surrounding tissue makes muscle cells prone to contraction-induced detachment: upon muscle contraction the cells detach and undergo apoptosis (programmed cell death). Even cells that do not enter the apoptotic programme suffer detrimental consequences such as increased autophagy and proteasome activity [16-21].

Tissue inflammation and degeneration induce an immune response attracting mainly macrophages to the injury site. Initially, macrophages present a pro-inflammatory phenotype secreting cytokines such as IL-6. They transition into an anti-inflammatory phenotype as the tissue recovers.

Table 1
Most common LAMA2-CMD mouse models.

Mouse model	Mutation	Laminin α2 chain	Severity	Reference
dy/dy	Unknown spontaneous mutation	Reduced levels	Moderate	[22]
dy^{2J}/dy^{2J}	Spontaneous mutation in LN domain	Reduced levels of truncated protein	Mild	[23]
dy^{3K}/dy^{3K}	Knock-out	Complete deficiency	Very severe	[24]

dy^{W}/dy^{W}	Knock-out	Very low levels of truncated protein	Severe	[25]

It is also worth mentioning the *mdx* mouse model of DMD, since it's the most studied mouse model of muscular dystrophy. It has a spontaneous mutation in the dystrophin gene resulting in a premature stop codon on exon 23 [26].

Table 2
Some pre-clinical therapeutic trials for LAMA2-CMD.

Approach	Mouse model	Outcome	Reference
Over-expression of human laminin α2 chain	dy^{W}/dy^{W}	Improved grip strength, fibre CSA normalisation and reduced fibrosis.	[25]
Over-expression of laminin α1 chain	dy^{3K}/dy^{3K}	Improved histology, bodyweight, locomotion, grip strength and lifespan; normalised CK levels.	[27; 28]
Apoptosis suppression	dy^{W}/dy^{W}, dy^{2J}/dy^{2J}	Improved bodyweight, grip strength, CSA distribution and lower serum CK levels.	[17; 29; 30]
Proteasome inhibition	dy^{3K}/dy^{3K}	Moderate improvement in fibre CSA, decreased apoptosis and fibrosis.	[20; 31]
Fibrosis and inflammation suppression	dy^{W}/dy^{W}, dy^{2J}/dy^{2J}	Improved bodyweight, grip strength and locomotion.	[32-34]
Linker proteins	dy^{W}/dy^{W}	Reduced fibrosis, central nucleation and inflammation, improved fibre CSA, myelination and locomotion.	[35; 36]

Fibrosis

Fibrosis is one of the most prominent and deleterious symptoms of LAMA2-CMD (as well as other muscular dystrophies). The progressive replacement of skeletal muscle for fibrous tissue renders it weaker and stiffer.

Fibrosis is (mainly) the result of net increases in collagen synthesis. Repeated degeneration/regeneration cycles lead to inflammation and incomplete ECM remodelling. Increased inflammation and TGF-β signalling in turn lead to excessive synthesis of collagen by fibroblasts, which progressively replaces muscle tissue. The reduced muscular content will need higher activation to meet force demands, making it prone to contraction-induced damage or detachment. The muscular milieu is thus in a feed-forward cycle that promotes fibrosis. Increased TGF-β activity not only promotes fibroblast proliferation but also impairs muscle growth. Myostatin is a TGF-β family member that negatively regulates muscle growth. Its inhibition in *mdx* mice improved histopathology and fibrosis [37; 38]. TGF-β induces fibrosis through canonical, i.e. Smad-mediated, and non-canonical pathways, e.g. MAPK. Another fibrosis-driving mechanism is the TGF-β-related renin-angiotensin system (RAS). Angiotensin (Ang) II is produced from AngI by the angiotensin-converting enzyme (ACE) 1. AngII can activate Smad and MAPK signalling to stimulate fibrosis. The ACE2 enzyme, however, catalyses the conversion of AngI into Ang-1-7, which in turn binds Mas receptors and elicits beneficial responses such as reduced oxidative stress and fibrosis [39].

Different attempts at blocking fibrosis in muscular dystrophies have yielded varying results, *mdx* mice being more responsive than LAMA2-CMD mouse models. Inhibiting TGF-β-related signalling is a clear course of action that has improved the *mdx* phenotype [40] and also LGMD. Ang-1-7 treatment inhibited TGF-β activity and consequently decreased miR-21 levels, which in turn reduced the number of fibroblasts and ECM synthesis [40]. Similarly, blocking angiotensin II receptor type 1 (AT1) in the dy^{W}/dy^{W} mouse model of LAMA2-CMD reduced fibrosis and inflammation, and improved body weight and muscle function [34]. In a similar study, Elbaz et al. [33] found that losartan, an AT1 antagonist, was efficient in reducing fibrosis in dy^{2J}/dy^{2J} mice by repressing TGF-β signalling. Nevo et al. [32] found improved histopathology in dy^{2J}/dy^{2J} mice with fibrosis inhibition. In their study, halofuginone treatment reduced collagen I synthesis by reducing phospho-Smad3 in fibroblasts surrounding centrally nucleated fibres.

Metabolic alterations

A number of metabolic alterations have been described in various muscular dystrophies, including redox unbalance, mitochondrial dysfunction and amino-acid and ion metabolism. Our group has shown altered gene expression and mitochondrial function in LAMA2-CMD patient cells [21].

We have also previously found that a high percentage (~40%) of differentially abundant proteins in dy^{3K}/dy^{3K} muscle are related to metabolism [41; 42] Therefore, metabolic alterations could potentially be disease-driving mechanisms or therapeutic targets. In the present work we assessed if metformin, the most widely used anti-diabetes drug, could improve muscle histology and function in the dy^{2J}/dy^{2J} mouse model of LAMA2-CMD.

MicroRNAs

MicroRNAs (miRNAs) are a class of small non-coding RNAs that have a regulatory role on mRNA translation. They work by base-complementarity to induce mRNA degradation or translation repression; i.e. they are post-transcriptional repressors [43].

MiRNAs are first transcribed in the nucleus as primary miRNAs (pri-miRNA), a multiple stem (or hairpin) loop structure that is hundreds of nucleotides in length. Already in the nucleus these are cleaved into precursor miRNAs (pre-miRNAs) with a single stem loop by a complex whose main components are Drosha and DGCR8. Pre-miRNAs are exported to the cytoplasm by exportin 5, where they're cleaved by DICER, yielding a double-stranded RNA. One of the strands will be incorporated into the RNA-induced silencing complex (RISC) to exert its biological function; this is the mature miRNA. The miRNA serves as a guiding template that drives RISC towards target mRNAs (figure 3). Nucleotides 2-8 on the 5'-end on a mature miRNA are called the seed. This region is the main factor in miRNA target recognition and specificity [43; 44].

Despite their non-coding nature, miRNAs can be located anywhere on the genome. Many miRNAs are within protein-coding regions, usually on introns, and are thus transcribed along with the host gene. MiRNAs located on exons or introns may bypass processing by Drosha/DGCR8 and instead use the spliceosome for cleavage (figure 3). Alternatively, miRNAs are located between genes (intergenic miRNAs) and are regulated by their own promoters. Some miRNAs form clusters (polycistronic miRNAs) and are transcribed together; these include the most prominent skeletal muscle miRNAs, i.e. miR-1, miR-133, miR-206 [43].

Interestingly, miRNAs are also found in extracellular spaces. It's not well-understood how the export process works nor what's the purpose of it. One

clear possibility is that miRNAs are involved in cell-to-cell communication. In support of this, there isn't a direct correlation between the miRNA profiles of different fractions, i.e. intra- and extra-cellular, vesicular [45-47]. For example, some miRNAs are only found in secreted vesicles [48]. Their presence in extracellular space also opens up the possibility of their use as biomarkers. Given the short half-life of RNA, extracellular miRNAs are bound to protein complexes (e.g. HDL or Argonaute) or encapsulated in vesicles of various sizes (e.g. exosomes) for increased stability.

Initial interest in miRNA research was due to their involvement in disease, particularly cancer. Later, they were found to be dysregulated in various diseases, including muscular dystrophies. MiRNAs are ubiquitous regulators of a multitude of cellular processes, making them interesting therapeutic targets.

MiRNAs in myogenesis

Striated muscle has some miRNAs which are semi-specific to it or enriched in the tissue: these are called myomiRs. They are miR-1, miR-133a/b and miR-206, the classical myomiRs, as well as the newcomers miR-208a/b, miR-486 and miR-499. MiRNAs that have a larger than 20-fold expression in skeletal muscle compared to other tissues are termed muscle-specific, whereas lower than 20-fold expression is considered muscle-enriched [43; 49; 50].

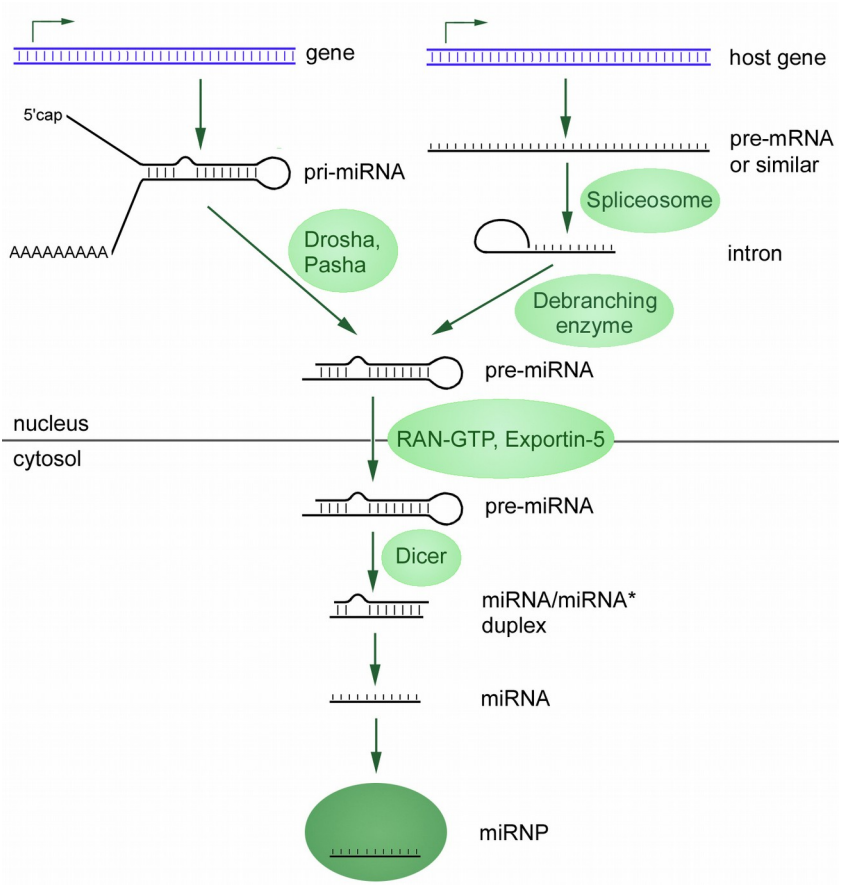

Figure 3.
MiRNA biogenesis. The upper-right branch depicts the alternative route when miRNAs have a host-gene.

MiRNAs influence processes important for myogenesis, i.e. proliferation, differentiation, fusion and migration. MyomiRs, amongst others, help fine-tune and orchestrate myogenesis [49; 51]. They are part of signalling pathways involved in cell survival and growth such as e.g. IGF-1/PI3K/Akt/mTOR, TGF-β and myostatin/FOXO [43; 51-53].

MiR-1, miR-206 and miR-133 are involved in perhaps the most fundamental aspect of myogenesis, i.e. they promote the adoption of myogenic cell lineages during embryogenesis by targeting genes that promote non-muscle cell paths. The expression of these miRNAs is under regulation of MRFs [50]
.

MiR-1 and miR-206 are largely involved in myoblast differentiation. These miRNAs differ in sequence by only four nucleotides in the 3' region, which translates into a great overlap in target-genes. They target Pax3 and Pax7 in progenitor cells to inhibit proliferation and stimulate differentiation [50]. MiR-1 and miR-206 affect myoblast differentiation by targeting HDAC4, a known repressor of muscle genes [50; 54; 55] . MiR-206 accomplishes this by inhibiting SMAD3 induction by TGF-β. They also target cell-cycle regulators such as cyclins and chromatin-remodelling factors. By doing so the miRNAs force cell cycle exit and thus halt proliferation.

The next myomiR, miR-133, is related to the previous two as they form bicistronic clusters: miR-1/miR-133a and miR-206/miR-133b [43; 50]. This means that their expression is related by some common regulatory elements. The role of miR-133 in myogenesis is more controversial, with evidence for the stimulation of both proliferation and differentiation. This suggests that the function of miR-133 may be context-dependent. MiR-133 stimulates proliferation by targeting serum response factor (SRF), an inducer of miR-1 and miR-206. Therefore, by targeting SRF miR-133 represses miR-1 and miR-206 and their anti-proliferative effects. On the other hand, miR-133 may inhibit proliferation by targeting FGFR1, a component of the MAPK pathway. Another MAPK component interacting with miR-133 is p38. It induces miR-133 and miR-1 expression, which in turn target SP1, an inducer of cyclin 1. Once again this has the effect of inducing cell cycle exit and halting proliferation. MiR-133 also interacts with the IGF-1 pathway: the latter induces miR-133a expression via MyoG, which targets IGF1R to down-regulate PI3K/Akt activity, thereby forming a negative feedback loop [50].

MiR-208a/b and miR-499 are encoded in intronic regions of myosin genes and thus play a role in fibre-type specification. MiR-208a is heart-specific and comes from the gene encoding Myh6, a fast myosin isoform. MiR-208b and miR-499, however, come from slow myosin genes (Myh7 and Myh7b, respectively) and are only expressed in slow fibres. These miRNAs have interesting target genes such as MAPK6 and myostatin [50].

MiR-486 is the newest myomiR and thus not so well-studied. Similarly to miR-1 and miR-206, it targets Pax7 to promote differentiation. MiR-486 stimulates PI3K/Akt activity: it directly targets PTEN and FOXO1, both negative regulators of the pathway [50].

MiRNAs in dystrophies

Muscular dystrophies generally arise from mutations in genes encoding DGC proteins. Some of these protein genes may themselves harbour miRNA genes, which could compound the problem. Furthermore, the absence of mRNAs/proteins may disrupt feedback/feed-forward loops that regulate miRNA expression and cellular homoeostasis. Thus, it's no surprise that miRNAs are dysregulated in muscular dystrophies [43; 56-58].

Our group showed that myomiRs, amongst others, are altered in LAMA2-CMD plasma and muscle [59]. A comprehensive study of miRNA expression in 10 types of muscular dystrophy was conducted by Eisenberg et al. [60], although LAMA2-CMD was not included. They showed that most dystrophies were associated with a specific set of dysregulated miRNAs, most of which were up-regulated in disease. This suggests that, despite common phenotypic observations of myofibre degeneration and fibrosis, there are disease-specific mechanisms driving these processes in different muscular dystrophies.

The most deleterious aspect of muscular dystrophies is fibrosis. MiR-21 promotes fibrosis in various diseases and tissues, such as muscle, lung, and kidney [61-64]. It is part of signalling networks involving TGF-β, MAPK and Akt/mTOR that upon stimulation promote fibroblast proliferation and collagen synthesis [65]. Interestingly, similar pathways act in macrophages to keep them in a pro-inflammatory state [66; 67]. Prolonged inflammation compromises the regeneration process thus aiding disease progression. MiR-21 levels are increased in LAMA2-CMD [59] but its genetic deletion does not improve muscle phenotype or function [68]. MiR-29 has the opposite action of miR-21, i.e. anti-fibrotic, and mimicking its activity significantly reduced collagen production in *mdx* myoblasts [63]. TGF-β can also stimulate miR-21 expression in fibroblasts via Smad signalling. TGF-β increases miR-21 levels post-transcriptionally by inducing Drosha activity and the processing of pri-miR-21 into pre-miR-21 [69]. One of miR-21 target genes is phosphatase and tensin homologue (PTEN), which is an inhibitor of Akt. Increased miR-21, therefore, stimulates Akt activity in fibroblasts to promote proliferation or survival.

MiRNAs as biomarkers

MiRNAs have been a hope in the search for biomarkers for muscular diseases. The classical myomiRs were the first proposal due to their higher

levels in patient and *mdx* sera [70-72]. They were thought to leak into circulation due to membrane damage or instability. However, their levels decrease with age and muscle mass, which could make their use difficult. At present it's still hard to interpret a response in the form of higher myomiR levels: does it mean increased muscle mass or necrosis? Zaharieva et al. [72] found that DMD patients with a milder disease course had higher myomiR levels than severe ones, suggesting that these miRNAs may serve as indicators of remaining muscle mass. Li et al. [58] further showed that miR-208 and miR-499 are also increased in serum of DMD patients. Furthermore, miR-133, miR-206, miR-208b and miR-499 were able to distinguish DMD from its milder variant BMD.

Muscular dystrophy patients usually die from cardio-respiratory failure. With this in mind Becker et al. [73] sought to find cardiomyopathy-related miRNA biomarkers. They found, amongst others, increased circulating miR-222, miR-26a and miR-378a in muscular dystrophy patients (DMD and BMD). What's more, these miRNAs were differentially expressed between patients with and without myocardial fibrosis. Another study involving DMD and BMD patients found increased miR-30c and miR-181a in serum [74] . Unlike the classical myomiRs, there was very low correlation between miRNA levels and age, which could make them more stable biomarkers. On the other hand, they found a trend for higher levels of miR-30c with better motor function (in DMD patients).

As we can note, the vast majority of biomarker studies for muscular dystrophies focuses on DMD. To date no study specifically looked for LAMA2-CMD biomarkers. We address this gap in our third article, where we explore urine miRNAs as non-invasive biomarkers of LAMA2-CMD disease progression.

MiRNAs as therapeutic targets

The ubiquitous role of miRNAs as regulators of biological processes also makes them interesting therapeutic targets. In this case, their action can either be blocked or enhanced. Blocking miRNA activity is usually achieved using anti-sense oligonucleotides (antagomiRs) or locked nucleic acids (LNA). These approaches work much like miRNAs themselves, i.e. by complementary-binding their targets and preventing their action; they're said to act as "sponges" that sequester miRNAs and prevent their action. Naturally, another approach is to remove the gene that encodes the miRNA.

One limitation of such method is unforeseen developmental changes. For an extensive review on miRNA inhibition methods see [75].

MiRNA over-expression has mostly been achieved by adeno-associated viral vectors (AAVs). However, system-wide expression is still infeasible due to liver toxicity [76]. Furthermore, AAVs pose a size-limit to the constructs they can deliver. For example dystrophin, the protein absent in DMD, is the largest protein in the human genome, making it unsuitable for AAV transfection [77].

Alexander et al. found that muscle-enriched miR-486 expression is lower in DMD and BMD patients, and its over-expression improves symptoms [78]. The beneficial effects were once again achieved by modulation of the PTEN/Akt pathway: miR-486 targets DOCK3, which in turn increases PTEN levels, an inhibitor of Akt. An interesting possibility that's rarely thought of is that miRNAs could also be exploited as adjunct therapy. For example, Cacchiarelli et al. [79] found that inhibition of miR-31 improved the efficiency of exon-skipping treatment in *mdx* mice. In this context miRNAs wouldn't be the main therapeutic targets but would improve the efficiency of other therapies.

Methods

Histology

Histology is a basic and vital technique in medical science. It allows the inspection of tissues and their properties under the light or electron microscope. Our group makes routine use of this technique to observe muscle fibres and their nuclei by means of haematoxylin and eosin staining (H&E). Haematoxylin binds DNA and colours the nuclei dark blue, which contrasts with the red/pink hues of the other (eosinophilic) structures [80] . We use H&E to assess overall muscle integrity and counting centrally located nuclei (articles II, III and IV).

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique that revolutionised biology. It was developed in the 80's by Kary Mullis and uses the ability of DNA polymerase to extend a nucleotide chain based on complementarity. Given that DNA polymerase can only extend an existing chain, a primer is used as an initial template. Primers are specific to the region that is to be amplified.

PCR works by thermal cycling: 1) denaturation – at 94 °C the DNA strands are separated, 2) annealing – at 54 °C the primers bind the single DNA strands and 3) extension – with the primers in place DNA polymerase can extend the chains. These 3 steps consstitute a single cycle, which will be repeated 30-40 times. Theoretically, every cycle doubles the DNA amount, the end result being millions of copies of the original DNA fragment [81] .

Quantitative PCR (qPCR) monitors the amount (amplification) of DNA during the reaction; it is also called real-time PCR (RT-PCR). However, the acronym RT-PCR usually refers to reverse transcription PCR, which is a

method for cloning regions of interest from their RNA. Thus RT-PCR reverse transcribes RNA into cDNA using reverse transcriptase.

In summary, PCR allows the absolute or relative quantification of gene expression. We have used qPCR in paper II to confirm the absence of miR-21 in relevant groups, as well as TGF-β expression.

Next-generation sequencing (NGS)

The term next-generation sequencing is an umbrella term for a few different technologies that have the aim of deciphering the sequence of nucleotide chains. Like PCR, NGS has revolutionised biomedical science in the last decade, allowing for cheaper and high-throughput experiments to be performed.

Sequencing machines are not yet capable of handling very long fragments, so the samples have to be fractioned into smaller fragments. The size of these will depend on the technology used for sequencing; e.g. Illumina sequencing can handle fragments ~150 nucleotides-long, Ion Torrent ~ 200bp and Roche 454 up to 1kb. Adapters are added after fragmentation; these are used to anchor the DNA templates to slides or beads and also aid the subsequent PCR reaction [82] .

For fragments to be sequenced with confidence, we need many of them. PCR is thus used to amplify the reads. The specifics of the PCR step may vary depending on the type of sequencing, e.g. Ion Torrent uses emulsion PCR. Regardless of that, the goal is to have many copies of a specific DNA template grouped in one place. The (PCR) amplification step is highly related to ‘sequence depth of coverage’ - the term is usually shortened to simply sequence depth or coverage. Sequence depth is the measure of how many times a given base in our sample has been sequenced. To be confident in our sequencing run we need sufficient coverage. The depth of coverage needed is determined by the type of sequencing (RNA-seq, ChIP-seq, miRNA-seq, etc), organism of origin and reference genome, for example [83].

The elongation of the PCR products and detection of incorporated nucleotides differ by manufacturer. Illumina sequencing binds the PCR product to a slide and floods it with nucleotides, DNA polymerase and a terminator which will limit the action of DNA polymerase to one single reaction. The nucleotides are colour-labelled and thus emit a specific signal

when added to the chain. A picture of the slide is taken indicating which nucleotides were added in each position. The terminator is removed and the cycle repeated. Based on the sequence of images (and colours) computers can determine the sequence of a fragment [82].

Roche 454 binds one DNA template per bead for amplification. The beads are then placed in a well with DNA polymerase and buffers. Next, the nucleotides are added sequentially, i.e. first A, then T, C and G, for example. A light signal is emitted when a nucleotide is incorporated, and the signal is proportional to the number of nucleotides added [82].

Differently from the two previous methods, which use optical signals, Ion Torrent/Proton makes use of pH changes to determine the fragment sequence. The addition of nucleotides by DNA polymerase releases a H^+ ion, one per added base. Like Roche 454, the chip is sequentially flooded with known nucleotides, and by measuring the drop in pH we know how many were added [82].

Ion Torrent sequencing was used in articles I and III to profile miRNA expression.

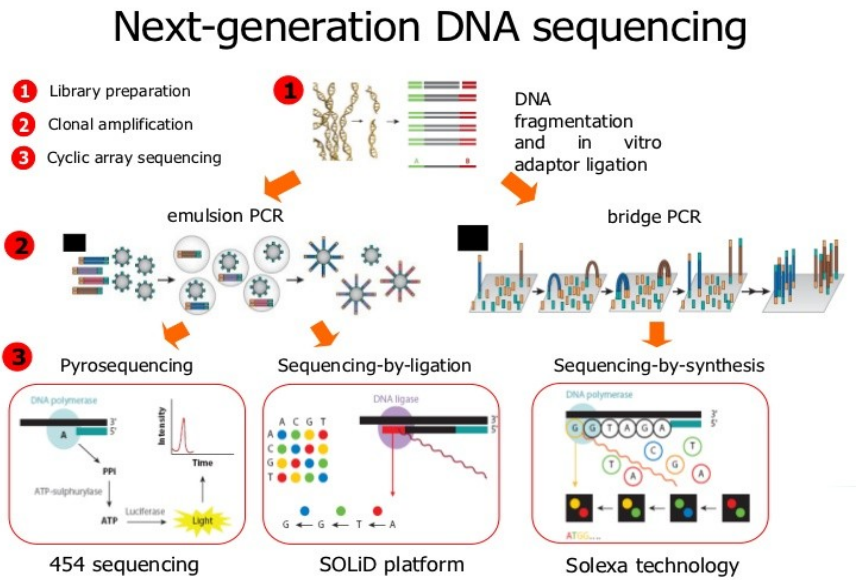

Figure 4.
Overview of some of the most common NGS platforms.

Main research questions

- What is the microRNA profile of laminin α2 chain-deficient muscle?
- Can the absence of pro-fibrotic miR-21 improve muscle phenotype and function in the dy^{3K}/dy^{3K} and dy^{2J}/dy^{2J} mouse models of LAMA2-CMD?
- Are urinary miRNAs potential biomarkers of LAMA2-CMD progression?
- Can metabolism be targeted to improve muscle function and phenotype in LAMA2-CMD mouse models? If so, can metformin be used for this purpose?

Results

Article I

In this work we have used next-generation sequencing to profile miRNAs in *dy^{3K}/dy^{3K}* muscle as an initial screening for our future studies. To make full use of the data we have integrated it with a previous (microarray) mRNA data set [41] from our group to get information about miRNA targets. We found 128 differentially expressed miRNAs in *dy^{3K}/dy^{3K}* muscle compared to wild-type: 70 up- and 58 down-regulated. Of the up-regulated miRNAs, miR-125a, miR-665 and miR-152 had more than 20 down-regulated targets. Conversely, amongst the down-regulated miRNAs with most up-regulated targets we find two myomiRs, namely miR-1a and miR-133a/b, with 17 and 15 targets, respectively. Moreover, expression of several dystromiRs such as miR-1, miR-29c, miR-133, miR-206, miR-223 and miR-499 was dysregulated also in *dy^{3K}/dy^{3K}* skeletal muscle. Furthermore, we also found other miRNAs that are common to various muscle disorders including DMD (e.g. miR-30c, miR-99b, miR-103, miR-125a, miR-195a, miR-214, miR-299a, miR-381, miR-501 and let-7e) [60]. Down-regulated genes targeted by multiple miRNAs include Arhgap28, Maf and S100pbp; and up-regulated targets include Bicc1, Bnc2, Dab2, Dclk1, Enpp1, Prune2 and Sox11. Gene set enrichment analysis of differentially expressed target genes found significant terms which include oxidative phosphorylation, metabolic pathways, protein and RNA metabolism, immunity, etc., in accordance with previous studies in our lab [21; 42], but also, to our knowledge, unrelated terms such as ‘Adrenergic signalling in cardiomyocytes’, and ‘AGE-RAGE signalling in diabetes’.

Article II

Considering the dysregulated miRNA profile observed in the first article and our previous studies, including pro-fibrotic miR-21, we explored the effects of its genetic deletion on skeletal muscle phenotype, function and fibrosis in LAMA2-CMD.

We found no improvement in body weight nor grip strength in either mouse model, despite a very slight reduction in collagen content in dy^{2J}/dy^{2J} double knock-out mice. There was a slight increase in central nucleation in dy^{3K}/dy^{3K} double knock-outs, which suggests increased regeneration; however, we couldn't confirm it with embryonic MHC staining (data not shown). In agreement with the lack of overt improvement, there was no significant change in TGF-β expression.

In summary we showed that the single deletion of miR-21 is not enough to reduce fibrosis in the dy^{2J}/dy^{2J} and dy^{3K}/dy^{3K} mouse models of LAMA2-CMD.

Article III

The lack of reliable biomarkers for myodegenerative diseases has been a limitation in the field for many years. Usually, patients have to undergo blood tests and tissue biopsies, which are invasive, especially for children. We tried to address this problem by exploring urinary miRNAs as reliable and non-invasive biomarkers for LAMA2-CMD. Our results show that already at 3 weeks of age, when no visible symptoms are present, five miRNAs are differentially expressed. At 4 weeks of age, when the first symptoms appear, we found the largest number of dysregulated miRNAs (18 microRNAs) and the only time point with down-regulated genes (in sick mice compared to control); a few muscle-enriched miRNAs are altered. At 6 weeks of age the symptoms are clear and myomiRs are amongst the differentially expressed miRNAs. We found 17 significant miRNAs that also include muscle-enriched and mitomiRs – i.e. mitochondria-related miRNAs.

Interestingly, we observed that male dy^{2J}/dy^{2J} mice have reduced body weight from 3 weeks onwards, compared to WT litter-mates, whilst females only differ at 6 weeks. Muscle function (assessed by grip strength) is lower and central nucleation higher from 4 weeks of age onward. Despite a trend at 4 weeks, collagen content is significantly elevated only at the 6 weeks time point. As expected, CK is higher in dystrophic animals at all time points analysed.

Article IV

A proteomics study performed by our group [42] found that most differentially abundant proteins between dy^{3K}/dy^{3K} and wild-type mice muscle were related to metabolic processes. We therefore chose to explore metabolism as a potential therapeutic target in a metformin intervention study. Six-week-old dy^{2J}/dy^{2J} and WT mice were treated with either metformin or water for 25 days. The analyses were factored on sex due to the different degree of muscle mass between males and females, and different response to disease observed in article III.

We found that metformin treatment improved grip strength in treated dy^{2J}/dy^{2J} compared to untreated, despite unaffected muscle weights. Energy efficiency was improved in treated dy^{2J}/dy^{2J} females, which resulted in increased weight gain in these mice. Central nucleation decreased in both dy^{2J}/dy^{2J} females and males in response to metformin, which suggests reduced muscle damage. We found a significant reduction of small fibres (500-1000 μm^2) in treated dy^{2J}/dy^{2J} females. White adipose tissue weight increased in both dy^{2J}/dy^{2J} females and males; in contrast, brown adipose tissue weight was reduced in dy^{2J}/dy^{2J} males.

Discussion

The main goal of my thesis was to explore potential therapies and biomarkers for LAMA2-CMD, particularly the potential of miRNAs. We've previously shown that miRNAs are dysregulated in LAMA2-CMD, specifically myomiRs, miR-21 and miR-223 [59]. To expand the scope of this analysis we have used miRNA-Seq to profile microRNAs in dy^{3K}/dy^{3K} muscle, which is presented in article I. We detected over 1500 miRNAs in dy^{3K}/dy^{3K} muscle, most of which were expressed at low levels. Of these, 128 were differentially expressed, 70 up- and 58 down-regulated in dystrophic muscle. The up-regulated miRNAs with most repressed target genes were miR-125a, miR-665, miR-152 and miR-501. Arhgap28 (targeted by miR-125a, miR-152 and miR-665) is an actin-interacting protein involved in cell contractility and ECM remodelling [84]. Given that the main function of laminin α2 chain is to connect the actin cytoskeleton of myotubes to the ECM, the repression of Arhgap28 is likely significant for LAMA2-CMD. Basically nothing is known about Maf (targeted by miR-125a, miR-152 and miR-665) in skeletal muscle. In other tissues, however, it interacts with p53 to influence apoptosis and cell cycle progression [85] and p53 has been demonstrated to mediate caspase-activation in LAMA2-CMD [86]. S100pbp (repressed; targeted by miR-125a, miR-501 and miR-665) and Bnc2 (up-regulated; targeted by miR-1a and miR-133a/b) are largely unknown genes except for some involvement in cancers. The function of Bicc1 (targeted by miR-20a and miR-133a/b) in skeletal muscle is mostly unexplored, but it is involved in muscle memory to loading: it was found hypo-methylated after a single bout of resistance exercise and its expression increased in response to reloading [87]. Dab2 (targeted by miR-20a and miR-133a/b) was linked to angiogenesis in facioscapulohumeral muscular dystrophy by interacting with FRG1 (FSHD region gene 1) [88]. More relevant to LAMA2-CMD, Dab2 mediates TGF-β induced fibronectin synthesis in fibroblast-like cell lines by activating JNK [89]. Dclk1 (targeted by miR-1a, miR-20a and miR-133a/b) and Prune2 (targeted by miR-1a, miR-20a, miR-22 and miR-133a/b) are genes involved in various forms of cancer, whilst Enpp1 (targeted by miR-1a, miR-20a, miR-22 and miR-133a/b) affects cardiac and vascular calcification. Sox11

(targeted by miR-1a, miR-22, miR-133a/b and miR-499), besides its involvement in cancers, affects nerve regeneration and CNS development. To better understand the potential role of these target genes we performed gene set enrichment analysis on them. These analyses look for over-represented genes related to a specific function, cellular component or process. Using the KEGG 2016 gene set we found interesting enriched terms such as oxidative phosphorylation, metabolic pathways and cardiac muscle contraction, suggesting that these processes are dysfunctional in LAMA2-CMD. We also found previously LAMA2-CMD-unrelated terms such as 'Adrenergic signalling in cardiomyocytes' and 'AGE-RAGE signalling in diabetes'.

One of our specific goals was to investigate if the genetic deletion of pro-fibrotic miR-21 would reduce fibrosis and improve muscle histology or function in the dy^{2J}/dy^{2J} and dy^{3K}/dy^{3K} mouse models of LAMA2-CMD. To our knowledge, this is the first study to address this question. We have shown that the genetic knock-out of miR-21 is not enough to reduce pathology, most likely due to miRNA redundancy. There are more than 130 miRNAs which share at least 50 targets with miR-21. At the moment miR-21's network in skeletal muscle is largely unknown, which makes it impossible for us to suggest potential candidates that could be taking its place. MicroRNA research still faces limitations such as the lack of validated targets; currently, Tarbase [90] doesn't list any miR-21 validated target in skeletal muscle. Furthermore, we didn't observe any reduction in TGF-β expression, which suggests that the overall inflammation status might be unchanged.

Another factor is different disease-driving mechanisms between LAMA2-CMD and DMD. Earlier studies were performed on the *mdx* mouse model of DMD, which presents a mild dystrophic phenotype compared to human DMD patients and our mouse models, especially the dy^{3K}/dy^{3K}. Furthermore, in DMD the whole DGC is missing from the sarcolemma, which is not the case in LAMA2-CMD. Muscle fibres in DMD are prone to contraction-induced damage whilst LAMA2-CMD leads to contraction-induced detachment. It seems that detachment is a strong signal for cell death and degeneration, leading to fibrosis.

The inability of the single deletion of miR-21 in reducing fibrosis in LAMA2-CMD makes us wonder about the deletion of multiple miRNAs, perhaps clusters or closely related miRNAs. At the moment this is a technical challenge: e.g. AAV vectors have limited capacity for carrying material, either for over-expression or gene editing [76; 77]. Also, genetic deletion of multiple genes may render embryos unviable or have undesired

developmental consequences. One potential limitation of this study is that the absence of miR-21, whilst perhaps beneficial for skeletal muscle fibrosis, could prove to be detrimental for other tissues. In that sense, post-natal RNA interference methods could be more a suitable way to dampen miR-21 activity instead of its complete genetic removal.

The third article explored the potential of urinary miRNAs as biomarkers of disease progression in LAMA2-CMD. We followed disease progression as routinely done with patients, i.e. muscle biopsies for histological inspection and blood concentration of creatine kinase, and also by miRNA sequencing. We found five miRNAs increased in the urine of dy^{2J}/dy^{2J} mice at 3 weeks of age, with no visible symptoms on histological sections. Although uninteresting from a diagnostic perspective, these miRNAs could hint at processes not analysed here, such as initial inflammatory response or immune cell infiltration. At this age, male dy^{2J}/dy^{2J} mice already have lower body weight than WT counterparts. At 4 weeks, with the first visible symptoms, we found the largest number of differentially expressed miRNAs (18 genes). It is also the only time point with down-regulated miRNAs in dy^{2J}/dy^{2J} compared to WT. At this stage dy^{2J}/dy^{2J} mice have increased central nucleation and decreased grip strength. Although there was a trend for higher collagen content in dystrophic animals, it did not reach statistical significance. At the latest time point we also found a large number of dysregulated miRNAs, seventeen genes. Interestingly, one miRNA that was down-regulated at 4 weeks is now up-regulated in dy^{2J}/dy^{2J} mice, namely miR-181a. It may prove to be an interesting candidate for further studies as it changes expression direction from 4 to 6 weeks of age, and it is a muscle-enriched mitochondria-associated miRNA. At 6 weeks of age the pathology is clear on histological sections and many of the differentially expressed miRNAs are either muscle-specific or muscle-enriched; collagen content is significantly higher in dy^{2J}/dy^{2J} mice and grip strength lower. Creatine kinase concentration was higher in dystrophic animals at all time points analysed.

Considering the minimal overlap of differentially expressed miRNAs between the time points analysed we propose that urine miRNAs could be used as biomarkers for LAMA2-CMD. Future studies should confirm our results with an orthogonal method, such as qPCR. Besides, future trials should also try to make use of patient samples, which will present challenges in the form of higher variance and data heterogeneity. One limitation of our study is that we had to pool urine samples for sequencing; it would be

interesting to repeat this study with one sample per subject, which should be possible with patient material.

In article IV we have explored a therapeutic intervention targeting metabolism. To do so we have used the FDA-approved drug metformin, the leading medication against type 2 diabetes. Metformin's mechanisms of action aren't completely understood yet, but it is known to act through AMPK to increase PGC-1α expression and mitochondrial content. It also inhibits gluconeogenesis in the liver and glucose uptake in the intestines; in skeletal muscle and adipose tissue it increases glucose uptake by increasing transport at the membrane [91]. These effects could counteract the decreased mitochondrial content and impaired energetic metabolism observed in patient cells [21].

The improved weight gain observed in treated dy^{2J}/dy^{2J} females seems to stem from non-muscle tissues, specifically, white adipose tissue. The reduction in brown adipose tissue in treated male dy^{2J}/dy^{2J} mice may suggest an effect of metformin on mitochondrial metabolism. Indeed, metformin uptake is substantial in brown adipose tissue, as recently shown by PET/CT [92] (Breining et al. 2018).

It is not clear what the cause of improved grip strength could be given that muscle weights did not change in response to metformin. We could speculate that, if the muscular component is not improved, then perhaps the neural one is. Metformin treatment might have improved motor neuron conductivity or NMJ stability. Future studies should address this possibility. The improved energy efficiency in dy^{2J}/dy^{2J} females might also have delayed fatigue in these mice.

A clear limitation of this study is the age at which treatment began, i.e. 6 weeks. In a clinical setting treatment would ensue as soon as the diagnosis is positive. It is therefore possible that metformin could alter disease progression and delay some of the symptoms.

Conclusions

The main finding of my thesis are:

There is substantial dysregulation in miRNA expression in dy^{3K}/dy^{3K} muscle. This is also highlighted by various target genes being differentially expressed, many of which are related to oxidative metabolism, muscle contraction, RNA, protein and amino-acid metabolism, amongst others. Some of the miRNAs with most target genes are myomiRs miR-1a, miR-133 and miR-499.

By itself, miR-21 knock-out is not enough to reduce fibrosis in LAMA2-CMD. Even in its absence, tissue degeneration is substantial, leading to progressive fibrosis and functional deficit.

MiRNAs are detectable in the urine of the dy^{2J}/dy^{2J} mouse model of LAMA2-CMD. They are potentially dysregulated already at 3 weeks of age, when no symptoms are visible. There is very little overlap in differentially expressed miRNAs throughout disease progression, and we thereby propose that future studies explore their potential.

Metformin yields some positive effects to dy^{2J}/dy^{2J} mice, especially females. Unfortunately, there was no improvement in body or muscle weights, indicating no major impact on skeletal muscle. Central nucleation was reduced in dy^{2J}/dy^{2J} mice with metformin treatment, suggesting reduced muscle degeneration. Most strikingly, metformin treatment improved grip strength in dystrophic mice.

Together, our studies show that LAMA2-CMD is a very difficult disease to tackle. Interventions that were beneficial for other muscular dystrophies show little or no impact on LAMA2-CMD. But not all is lost. Our results suggest that urinary miRNAs can indeed be used as biomarkers. Future studies should benefit from larger sample sizes and patient material. Metabolism-related features have been consistently altered in our recent investigations, indicating that metabolism is a logical therapeutic target to be pursued. Although metformin did not increase body and muscle weights, it might have improved

neural function, leading to the increased grip strength observed. Besides, there are other compounds to be explored, such as anti-oxidant agents.

References

[1] Lee, D. E.; Brown, J. L.; Rosa-Caldwell, M. E.; Blackwell, T. A.; Perry, R. A.; Brown, L. A.; Khatri, B.; Seo, D.; Bottje, W. G.; Washington, T. A.; Wiggs, M. P.; Kong, B.-W. and Greene, N. P. (2017). *Cancer cachexia-induced muscle atrophy: evidence for alterations in microRNAs important for muscle size*, Physiological Genomics 49 : 253-260.

[2] Cetrone, M.; Mele, A. and Tricarico, D. (2014). *Effects of the Antidiabetic Drugs on the Age-Related Atrophy and Sarcopenia Associated with Diabetes Type II.*, Current Diabetes Reviews 10 : 231-237.

[3] Hayot, M.; Rodriguez, J.; Vernus, B.; Carnac, G.; Jean, E.; Allen, D.; Goret, L.; Obert, P.; Candau, R. and Bonnieu, A. (2011). *Myostatin up-regulation is associated with the skeletal muscle response to hypoxic stimuli*, Molecular and Cellular Endocrinology 332 : 38-47.

[4] Zatz, M.; Vainzof, M. and Passos-Bueno, M. R. (2001). *Serum Creatine Kinase in Progressive Muscular Dystrophies*. In: (Ed.), *Muscular Dystrophy*, Springer Nature.

[5] Bentzinger, C. F.; Wang, Y. X. and Rudnicki, M. A. (2012). *Building Muscle: Molecular Regulation of Myogenesis*, Cold Spring Harbor Perspectives in Biology 4 : a008342-a008342.

[6] Fujita, R. and Crist, C. (2018). *Translational Control of the Myogenic Program in Developing, Regenerating, and Diseased Skeletal Muscle*. In: (Ed.), *Current Topics in Developmental Biology*, Elsevier.

[7] Gillies, A. R. and Lieber, R. L. (2011). *Structure and function of the skeletal muscle extracellular matrix*, Muscle & Nerve : n/a-n/a.

[8] de Rezende Pinto, W. B. V.; de Souza, P. V. S. and Oliveira, A. S. B. (2015). *Normal muscle structure, growth, development, and regeneration*, Current Reviews in Musculoskeletal Medicine 8 : 176-181.

[9] Pozzi, A.; Yurchenco, P. D. and Iozzo, R. V. (2017). *The nature and biology of basement membranes*, Matrix Biology 57-58 : 1 - 11.

[10] Sanes, J. R. (2003). *The Basement Membrane/Basal Lamina of Skeletal Muscle*, Journal of Biological Chemistry 278 : 12601-12604.

[11] Holmberg, J. and Durbeej, M. (2013). *Laminin-211 in skeletal muscle function*, Cell Adhesion and Migration 7 : 1-11.

[12] Lowe, J. S. and Anderson, P. G. (2015). *Support Cells and the Extracellular Matrix*. In: (Ed.), *Stevens Lowes Human Histology*, Elsevier.

[13] Durbeej, M. and Campbell, K. P. (2002). *Muscular dystrophies involving the dystrophin–glycoprotein complex and an overview of current mouse models*, Genetics of Disease 12 : 349-361.

[14] Lim, L. E. and Campbell, K. P. (1998). *The sarcoglycan complex in limb–girdle muscular dystrophy*, Current Opinion in Neurology 11 : 443-452.

[15] Murphy, A. P. and Straub, V. (2015). *The Classification, Natural History and Treatment of the Limb Girdle Muscular Dystrophies*, Journal of Neuromuscular Diseases 2 : S7–S19.

[16] Durbeej, M. (2015). *Laminin-upalpha2~Chain-Deficient Congenital Muscular Dystrophy*, Current Topics in Membranes 76 : 31-60.

[17] Girgenrath, M.; Dominov, J. A.; Kostek, C. A. and Miller, J. B. (2004). *Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy*, The Journal of Clinical Investigation 114 : 1635-1639.

[18] Meinen, S.; Lin, S.; Thurnherr, R.; Erb, M.; Meier, T. and Rüegg, M. A. (2011). *Apoptosis inhibitors and mini-agrin have additive benefits in congenital muscular dystrophy mice*, EMBO Molecular Medicine 3 : 465–479.

[19] Yamauchi, J.; Kumar, A.; Duarte, L.; Mehuron, T. and Girgenrath, M. (2013). *Triggering regeneration and tackling apoptosis: a combinatorial approach to treating congenital muscular dystrophy type 1~A*, Human Molecular Genetics 22 : 4306–4317.

[20] Körner, Z.; Fontes-Oliveira, C. C.; Holmberg, J.; Carmignac, V. and Durbeej, M. (2014). *Bortezomib Partially Improves Laminin a2 Chain-Deficient Muscular Dystrophy*, The American Journal of Pathology 184 : 1518-1528.

[21] Fontes-Oliveira, C. C.; Steinz, M.; Schneiderat, P.; Mulder, H. and Durbeej, M. (2017). *Bioenergetic Impairment in Congenital Muscular Dystrophy Type 1A and Leigh Syndrome Muscle Cells*, Scientific Reports 7 : 45272.

[22] Sunada, Y.; Bernier, S. M.; Kozak, C. A.; Yamada, Y. and Campbell, K. P. (1994). *Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus.*, Journal of Biological Chemistry 269 : 13729-13732.

[23] Xu, H.; Wu, X.-R.; Wewer, U. M. and Engvall, E. (1994). *Murine muscular dystrophy caused by a mutation in the laminin upalpha2 (Lama2) gene*, Nature Genetics 8 : 297-302.

[24] Miyagoe, Y.; Hanaoka, K.; Nonaka, I.; Hayasaka, M.; Nabeshima, Y.; Arahata, K.; Nabeshima, Y.-i. and Takeda, S. (1997). *Laminin α2 chain-null mutant mice by targeted disruption of the Lama2 gene: a new model of merosin (laminin 2)-deficient congenital muscular dystrophy*, FEBS Letters 415 : 33-39.

[25] Kuang, W.; Xu, H.; Vachon, P. H.; Liu, L.; Loechel, F.; Wewer, U. M. and Engvall, E. (1998). *Merosin-deficient congenital muscular dystrophy. Partial*

genetic correction in two mouse models., The Journal of Clinical Investigation 102 : 844-852.

[26] Manning, J. and O'Malley, D. (2015). *What has the mdx mouse model of duchenne muscular dystrophy contributed to our understanding of this disease?*, Journal of Muscle Research and Cell Motility 36 : 155-167.

[27] Gawlik, K.; Miyagoe-Suzuki, Y.; Ekblom, P.; Takeda, S. and Durbeej, M. (2004). *Laminin α1 chain reduces muscular dystrophy in laminin α2 chain deficient mice*, Human Molecular Genetics 13 : 1775-1784.

[28] Gawlik, K. I. and Durbeej, M. (2010). *TRANSGENIC OVEREXPRESSION OF LAMININ α1 CHAIN IN LAMININ α2 CHAIN–DEFICIENT MICE RESCUES THE DISEASE THROUGHOUT THE LIFESPAN*, Muscle Nerve 42 : 30-37.

[29] Erb, M.; Meinen, S.; Barzaghi, P.; Sumanovski, L. T.; Courdier-Früh, I.; Rüegg, M. A. and Meier, T. (2009). *Omigapil Ameliorates the Pathology of Muscle Dystrophy Caused by Laminin-α2 Deficiency*, Journal of Pharmacology and Experimental Therapeutics 331 : 787-795.

[30] Yu, X. and Zuo, Q. (2013). *MicroRNAs in the regeneration of skeletal muscle~*, Frontiers in Bioscience 18 : 608.

[31] Carmignac, V.; Quéré, R. and Durbeej, M. (2011). *Proteasome inhibition improves the muscle of laminin a2 chain-deficient mice*, Human Molecular Genetics 20 : 541-552.

[32] Nevo, Y.; Halevy, O.; Genin, O.; Moshe, I.; Turgeman, T.; Harel, M.; Biton, E.; Reif, S. and Pines, M. (2010). *Fibrosis inhibition and muscle histopathology improvement in laminin-upalpha2-deficient mice*, Muscle & Nerve 42 : 218-229.

[33] Elbaz, M.; Yanay, N.; Aga-Mizrachi, S.; Brunschwig, Z.; Kassis, I.; Ettinger, K.; Barak, V. and Nevo, Y. (2012). *Losartan, a therapeutic candidate in congenital muscular dystrophy: Studies in the dy2J/dy2J Mouse*, Annals of Neurology 71 : 1531-8249699-708.

[34] Meinen, S.; Lin, S. and Ruegg, M. A. (2012). *Angiotensin II type 1 receptor antagonists alleviate muscle pathology in the mouse model for laminin-α2-deficient congenital muscular dystrophy (MDC1A)*, Skeletal Muscle 2.

[35] Moll, J.; Barzaghi, P.; Lin, S.; Bezakova, G.; Lochmüller, H.; Engvall, E.; Müller, U. and Ruegg, M. A. (2001). *An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy*, Nature 413 : 302-307.

[36] Reinhard, J. R.; Lin, S.; McKee, K. K.; Meinen, S.; Crosson, S. C.; Sury, M.; Hobbs, S.; Maier, G.; Yurchenco, P. D. and Rüegg, M. A. (2017). *Linker proteins restore basement membrane and correct LAMA2-related muscular dystrophy in mice*, Science Translational Medicine 9.

[37] Li, Z. B.; Kollias, H. D. and Wagner, K. R. (2008). *Myostatin Directly Regulates Skeletal Muscle Fibrosis*, Journal of Biological Chemistry 283 : 19371-19378.

[38] Li, L.; Sarver, A. L.; Alamgir, S. and Subramanian, S. (2012). *Downregulation of microRNAs miR-1, -206 and -29 -stabilizes PAX3 and CCND2 expression in rhabdomyosarcoma*, Laboratory Investigation 92 : 571-583.

[39] Smith, L. R. and Barton, E. R. (2018). *Regulation of fibrosis in muscular dystrophy*, Matrix Biology .

[40] Acuna, M. J.; Pessina, P.; Olguin, H.; Cabrera, D.; Vio, C. P.; Bader, M.; Munoz-Canoves, P.; Santos, R. A.; Cabello-Verrugio, C. and Brandan, E. (2013). *Restoration of muscle strength in dystrophic muscle by angiotensin-1-7 through inhibition of TGF-~ signalling*, Human Molecular Genetics 23 : 1237-1249.

[41] Häger, M.; Bigotti, M. G.; Meszaros, R.; Carmignac, V.; Holmberg, J.; Allamand, V.; Akerlund, M.; Kalamajski, S.; Brancaccio, A.; Mayer, U. and Durbeej, M. (2008). *Cib2 binds integrin alpha7Bbeta1D and is reduced in laminin alpha2 chain-deficient muscular dystrophy*, The Journal of biological chemistry 283 : 24760—24769.

[42] de Oliveira, B. M.; Matsumura, C. Y.; Fontes-Oliveira, C. C.; Gawlik, K. I.; Acosta, H.; Wernhoff, P. and Durbeej, M. (2014). *Quantitative Proteomic Analysis Reveals Metabolic Alterations, Calcium Dysregulation, and Increased Expression of Extracellular Matrix Proteins in Laminin α2 Chain–deficient Muscle*, Mol Cell Proteomics 13 : 3001-3013.

[43] Zacharewicz, E.; Lamon, S. and Russell, A. P. (2013). *MicroRNAs in skeletal muscle and their regulation with exercise, ageing and disease*, Frontiers in Physiology 4.

[44] Bartel, D. P. (2009). *MicroRNAs: Target Recognition and Regulatory Functions*, Cell 136 : 215-233.

[45] Roberts, T. C.; Godfrey, C.; McClorey, G.; Vader, P.; Briggs, D.; Gardiner, C.; Aoki, Y.; Sargent, I.; Morgan, J. E. and Wood, M. J. (2013). *Extracellular microRNAs are dynamic non-vesicular biomarkers of muscle turnover*, Nucleic Acids Research 1.

[46] Matsuzaka, Y.; Tanihata, J.; Komaki, H.; Ishiyama, A.; Oya, Y.; Rüegg, U.; Takeda, S.-i. and Hashido, K. (2016). *Characterization and Functional Analysis of Extracellular Vesicles and Muscle-Abundant miRNAs (miR-1, miR-133a, and miR-206) in C2C12 Myocytes and mdx Mice*, PLOS ONE 11 : e0167811.

[47] Wang, H. and Wang, B. (2016). *Extracellular vesicle microRNAs mediate skeletal muscle myogenesis and disease (Review)*, Biomedical Reports .

[48] Redis, R. S.; Calin, S.; Yang, Y.; You, M. J. and Calin, G. A. (2012). *Cell-to-cell miRNA transfer: From body homeostasis to therapy*, Pharmacology & Therapeutics 136 : 169-174.

[49] Diniz, G. P. and Wang, D. (2016). . In: (Ed.), *Regulation of Skeletal Muscle by microRNAs*, American Cancer Society.

[50] Horak, M.; Novak, J. and Bienertova-Vasku, J. (2016). *Muscle-specific microRNAs in skeletal muscle development*, Developmental Biology 410 : 1-13.

[51] McCarthy, J. J. (2014). *microRNA and skeletal muscle function: novel potential roles in exercise, diseases, and aging*, Frontiers in Physiology 5.

[52] Meyer, S. U.; Sass, S.; Mueller, N. S.; Krebs, S.; Bauersachs, S.; Kaiser, S.; Blum, H.; Thirion, C.; Krause, S.; Theis, F. J. and Pfaffl, M. W. (2015). *Integrative Analysis of MicroRNA and mRNA Data Reveals an Orchestrated Function of MicroRNAs in Skeletal Myocyte Differentiation in Response to TNF-upalpha or IGF1*, PLOS ONE 10 : e0135284.

[53] Meyer, S. U.; Thirion, C.; Polesskaya, A.; Bauersachs, S.; Kaiser, S.; Krause, S. and Pfaffl, M. W. (2015). *TNF-upalpha and IGF1 modify the microRNA signature in skeletal muscle cell differentiation*, Cell Communication and Signaling 13 : 4.

[54] Chen, J.-F.; Mandel, E. M.; Thomson, J. M.; Wu, Q.; Callis, T. E.; Hammond, S. M.; Conlon, F. L. and Wang, D.-Z. (2006). *The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation*, Nature Genetics 38 : 228–233.

[55] Goljanek-Whysall, K.; Pais, H.; Rathjen, T.; Sweetman, D.; Dalmay, T. and Münsterberg, A. (2012). *Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblast differentiation*, Journal of Cell Science 125 : 3590-3600.

[56] Erriquez, D.; Perini, G. and Ferlini, A. (2013). *Non-Coding RNAs in Muscle Dystrophies*, International Journal of Molecular Sciences 14 : 19681–19704.

[57] Morlando, M.; Rosa, A.; Caffarelli, E.; Fatica, A. and Bozzoni, I. (2013). *Non Coding RNA in Muscle Differentiation and Disease*, MicroRNA 2 : 91-101.

[58] Li, X.; Li, Y.; Zhao, L.; Zhang, D.; Yao, X.; Zhang, H.; Wang, Y.-c.; Wang, X.-y.; Xia, H.; Yan, J. and et al. (2014). *Circulating Muscle-specific miRNAs in Duchenne Muscular Dystrophy Patients*, Mol Ther Nucleic Acids 3 : e177.

[59] Holmberg, J.; Alajbegovic, A.; Gawlik, K. I.; Elowsson, L. and Durbeej, M. (2014). *Laminin α2 Chain-Deficiency is Associated with microRNA Deregulation in Skeletal Muscle and Plasma*, Frontiers in Aging Neuroscience 6.

[60] Eisenberg, I.; Eran, A.; Nishino, I.; Moggio, M.; Lamperti, C.; Amato, A. A.; Lidov, H. G.; Kang, P. B.; North, K. N.; Mitrani-Rosenbaum, S.; Flani, K. M.; Neely, L. A.; Whitney, D.; Beggs, A. H.; Kohane, I. S. and Kunkel, L. M. (2007). *Distinctive patterns of microRNA expression in primary muscular disorders*, PNAS 104 : 17016-17021.

[61] Patrick, D. M.; Montgomery, R. L.; Qi, X.; Obad, S.; Kauppinen, S.; Hill, J. A.; van Rooij, E. and Olson, E. N. (2010). *Stress-dependent cardiac remodeling*

occurs in the absence of microRNA-21 in mice, The Journal of Clinical Investigation 120 : 3912-3916.

[62] Ardite, E.; Perdiguero, E.; Vidal, B.; Gutarra, S.; Serrano, A. L. and Muñoz-Cánoves, P. (2012). *PAI-1–regulated miR-21 defines a novel age-associated fibrogenic pathway in muscular dystrophy*, The Journal of Cell Biology 196 : 163-175.

[63] Zanotti, S.; Gibertini, S.; Curcio, M.; Savadori, P.; Pasanisi, B.; Morandi, L.; Cornelio, F.; Mantegazza, R. and Mora, M. (2015). *Opposing roles of miR-21 and miR-29 in the progression of fibrosis in Duchenne muscular dystrophy*, Biochimica et Biophysica Acta 1852 : 1451-1464.

[64] Loboda, A.; Sobczak, M.; Jozkowicz, A. and Dulak, J. (2016). *TGF-β1/Smads and miR-21 in Renal Fibrosis and Inflammation*, Mediators of Inflammation 2016 : 1-12.

[65] Thum, T.; Gross, C.; Fiedler, J.; Fischer, T.; Kissler, S.; Bussen, M.; Galuppo, P.; Just, S.; Rottbauer, W.; Frantz, S.; Castoldi, M.; Soutschek, J.; Koteliansky, V.; Rosenwald, A.; Basson, M. A.; Licht, J. D.; Pena, J. T. R.; Rouhanifard, S. H.; Muckenthaler, M. U.; Tuschl, T.; Martin, G. R.; Bauersachs, J. and Engelhardt, S. (2008). *MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts*, Nature 456 : 980-984.

[66] Perdiguero, E.; Sousa-Victor, P.; Ruiz-Bonilla, V.; Jard, M.; Caelles, C.; Serrano, A. L. and Muñoz-Cánoves, P. (2011). *p38/MKP-1–regulated AKT coordinates macrophage transitions and resolution of inflammation during tissue repair*, J Cell Biol 195 : 307-322.

[67] Perdiguero, E.; Kharraz, Y.; Serrano, A. L. and Muñoz-Cánoves, P. (2012). *MKP-1 coordinates ordered macrophage-phenotype transitions essential for stem cell-dependent tissue repair*, Cell Cycle 11 : 877-886.

[68] Oliveira, B. M. S.; Durbeej, M. and Holmberg, J. (2017). *Absence of microRNA-21 does not reduce muscular dystrophy in mouse models of LAMA2-CMD*, PLOS ONE 12 : e0181950.

[69] Davis, B. N.; Hilyard, A. C.; Lagna, G. and Hata, A. (2008). *SMAD proteins control DROSHA-mediated microRNA maturation*, Nature 454 : 56-61.

[70] Cacchiarelli, D.; Legnini, I.; Martone, J.; Cazzella, V.; D'Amico, A.; Bertini, E. and Bozzoni, I. (2011). *miRNAs as serum biomarkers for Duchenne muscular dystrophy*, EMBO Molecular Medicine 3 : 258-265.

[71] Mizuno, H.; Nakamura, A.; Aoki, Y.; Ito, N.; Kishi, S.; Yamamoto, K.; Sekiguchi, M.; Takeda, S. and Hashido, K. (2011). *Identification of Muscle-Specific MicroRNAs in Serum of Muscular Dystrophy Animal Models: Promising Novel Blood-Based Markers for Muscular Dystrophy*, PLoS ONE 6 : e18388.

[72] Zaharieva, I. T.; Calissano, M.; Scoto, M.; Preston, M.; Cirak, S.; Feng, L.; Collins, J.; Kole, R.; Guglieri, M.; Straub, V.; Bushby, K.; Ferlini, A.; Morgan,

J. E. and Muntoni, F. (2013). *Dystromirs as Serum Biomarkers for Monitoring the Disease Severity in Duchenne Muscular Dystrophy*, PLoS ONE 8 : e80263.

[73] Becker, S.; Florian, A.; Patrascu, A.; Rösch, S.; Waltenberger, J.; Sechtem, U.; Schwab, M.; Schaeffeler, E. and Yilmaz, A. (2016). *Identification of cardiomyopathy associated circulating miRNA biomarkers in patients with muscular dystrophy using a complementary cardiovascular magnetic resonance and plasma profiling approach*, Journal of Cardiovascular Magnetic Resonance 18.

[74] Llano-Diez, M.; Ortez, C. I.; Gay, J. A.; Álvarez-Cabado, L.; Jou, C.; Medina, J.; Nascimento, A. and Jimenez-Mallebrera, C. (2017). *Digital PCR quantification of miR-30c and miR-181a as serum biomarkers for Duchenne muscular dystrophy*, Neuromuscular Disorders 27 : 15-23.

[75] Beavers, K. R.; Nelson, C. E. and Duvall, C. L. (2015). *MiRNA inhibition in tissue engineering and regenerative medicine*, Advanced Drug Delivery Reviews 88 : 123-137.

[76] Colella, P.; Ronzitti, G. and Mingozzi, F. (2018). *Emerging Issues in AAV-Mediated In~Vivo Gene Therapy*, Molecular Therapy - Methods & Clinical Development 8 : 87-104.

[77] Naso, M. F.; Tomkowicz, B.; Perry, W. L. and Strohl, W. R. (2017). *Adeno-Associated Virus (AAV) as a Vector for Gene Therapy*, BioDrugs 31 : 317-334.

[78] Alexander, M. S.; Casar, J. C.; Motohashi, N.; Vieira, N. M.; Eisenberg, I.; Marshall, J. L.; Gasperini, M. J.; Lek, A.; Myers, J. A.; Estrella, E. A.; Kang, P. B.; Shapiro, F.; Rahimov, F.; Kawahara, G.; Widrick, J. J. and Kunkel, L. M. (2014). *MicroRNA-486-dependent modulation of DOCK3/PTEN/AKT signaling pathways improves muscular dystrophy-associated symptoms*, Journal of Clinical Investigation 124 : 2651-2667.

[79] Cacchiarelli, D.; Incitti, T.; Martone, J.; Cesana, M.; Cazzella, V.; Santini, T.; Sthandier, O. and Bozzoni, I. (2011). *miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy*, EMBO reports 12 : 136-141.

[80] Feldman, A. T. and Wolfe, D. (2014). *Tissue Processing and Hematoxylin and Eosin Staining*. In: (Ed.), *Histopathology*, Springer New York.

[81] Eeles, R. A.; Warren, W. and Stamps, A. (1992). *The PCR revolution*, European Journal of Cancer 28 : 289-293.

[82] Heather, J. M. and Chain, B. (2016). *The sequence of sequencers: The history of sequencing DNA*, Genomics 107 : 1-8.

[83] Muzzey, D.; Evans, E. A. and Lieber, C. (2015). *Understanding the Basics of NGS: From Mechanism to Variant Calling*, Current Genetic Medicine Reports 3 : 158-165.

[84] Yeung, C.-Y. C.; Taylor, S. H.; Garva, R.; Holmes, D. F.; Zeef, L. A.; Soininen, R.; Boot-Handford, R. P. and Kadler, K. E. (2014). *Arhgap28 Is a RhoGAP*

that Inactivates RhoA and Downregulates Stress Fibers, PLoS ONE 9 : e107036.

[85] Hale, T. K.; Myers, C.; Maitra, R.; Kolzau, T.; Nishizawa, M. and Braithwaite, A. W. (2000). *Maf Transcriptionally Activates the Mousep53Promoter and Causes a p53-dependent Cell Death*, Journal of Biological Chemistry 275 : 17991-17999.

[86] Yoon, S.; Beermann, M. L.; Yu, B.; Shao, D.; Bachschmid, M. and Miller, J. B. (2018). *Aberrant Caspase Activation in Laminin-α2-Deficient Human Myogenic Cells is Mediated by p53 and Sirtuin Activity*, Journal of Neuromuscular Diseases 5 : 59-73.

[87] Seaborne, R. A.; Strauss, J.; Cocks, M.; Shepherd, S.; O'Brien, T. D.; van Someren, K. A.; Bell, P. G.; Murgatroyd, C.; Morton, J. P.; Stewart, C. E. and Sharples, A. P. (2018). *Human Skeletal Muscle Possesses an Epigenetic Memory of Hypertrophy*, Scientific Reports 8.

[88] Wuebbles, R. D.; Hanel, M. L. and Jones, P. L. (2009). *FSHD region gene 1 (FRG1) is crucial for angiogenesis linking FRG1 to facioscapulohumeral muscular dystrophy-associated vasculopathy*, Disease Models & Mechanisms 2 : 267-274.

[89] Hocevar, B. A.; Prunier, C. and Howe, P. H. (2005). *Disabled-2 (Dab2) Mediates Transforming Growth Factor upbeta (TGFupbeta)-stimulated Fibronectin Synthesis through TGFupbeta-activated Kinase 1 and Activation of the JNK Pathway*, Journal of Biological Chemistry 280 : 25920-25927.

[90] Vlachos, I. S.; Paraskevopoulou, M. D.; Karagkouni, D.; Georgakilas, G.; Vergoulis, T.; Kanellos, I.; Anastasopoulos, I.-L.; Maniou, S.; Karathanou, K.; Kalfakakou, D.; Fevgas, A.; Dalamagas, T. and Hatzigeorgiou, A. G. (2014). *DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions*, Nucleic Acids Research 43 : D153-D159.

[91] Viollet, B. and Foretz, M. (2013). *Revisiting the mechanisms of metformin action in the liver*, Annales d'Endocrinologie 74 : 123-129.

[92] Breining, P.; Jensen, J. B.; Sundelin, E. I.; Gormsen, L. C.; Jakobsen, S.; Busk, M.; Rolighed, L.; Bross, P.; Fernandez-Guerra, P.; Markussen, L. K.; Rasmussen, N. E.; Hansen, J. B.; Pedersen, S. B.; Richelsen, B. and Jessen, N. (2018). *Metformin targets brown adipose tissue in vivo and reduces oxygen consumption in vitro*, Diabetes, Obesity and Metabolism .