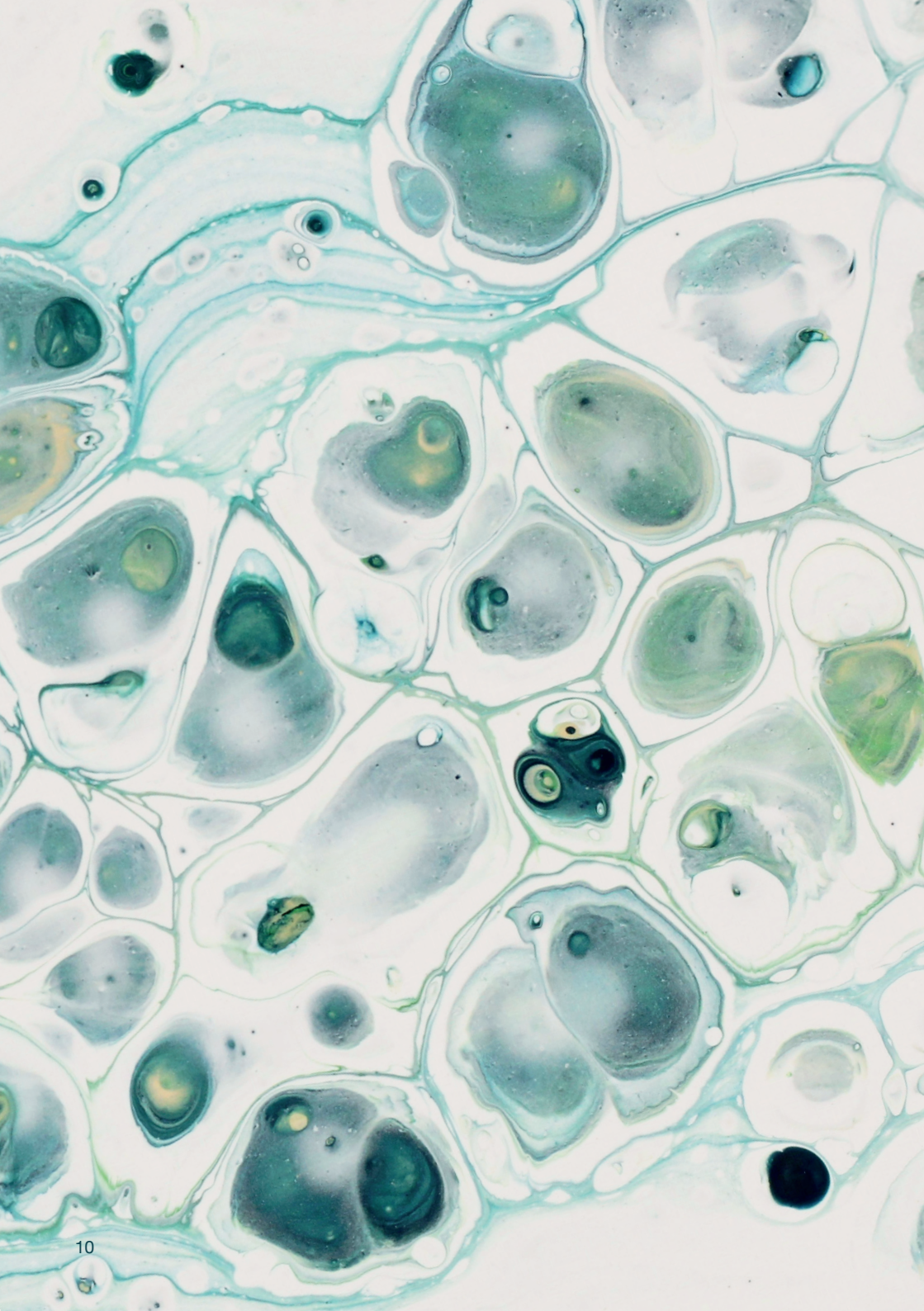
Universiteit Leiden
The Netherlands

Mapping the unseen to uncover the unknown: spatial analysis of neuromuscular disorders

Heezen, L.G.M.



The beginning of all wisdom is wonder

- Aristotle

Chapter 1

Introduction

Neuromuscular disorders (NMDs) consist of a wide range of diseases amongst which: congenital myopathies, hereditary neuropathies, inflammatory and metabolic myopathies, myasthenia gravis, myotonic syndromes, spinal muscular atrophies and muscular dystrophies (Laing, 2012). However, the etiology of these disorders vary, they can be either genetic or acquired conditions. Where an inflammatory myopathy such as polymyositis is thought to be caused by an autoimmune response, muscular dystrophies such as Duchenne muscular dystrophy are inherited or caused by spontaneous gene mutations (Laing, 2012).

1.1 GENETIC BASIS OF DYSTROPHINOPATHIES

This thesis focuses on Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy (DMD), commonly referred to as dystrophinopathies. This is a group of muscular dystrophies caused by pathogenic variants in the dystrophin (*DMD*) gene that is located on the X chromosome (Xp21 locus) (Aartsma et al., 2006). The gene is composed of multiple promoters, giving rise to three full-length isoforms (Dp427$_{c, m \text{ and } p}$) and multiple shorter isoforms (Dp260, Dp140, Dp116, Dp71 and Dp40) (Chelly et al., 1990; Górecki et al., 1992; Doorenweerd et al., 2017; D'Souza et al., 1995; Lidov, Selig and Kunkel, 1995; Byers, Ildov and Kunkel, 1993; Hugnot et al., 1992; Tinsley, Blake and Davies, 1993).

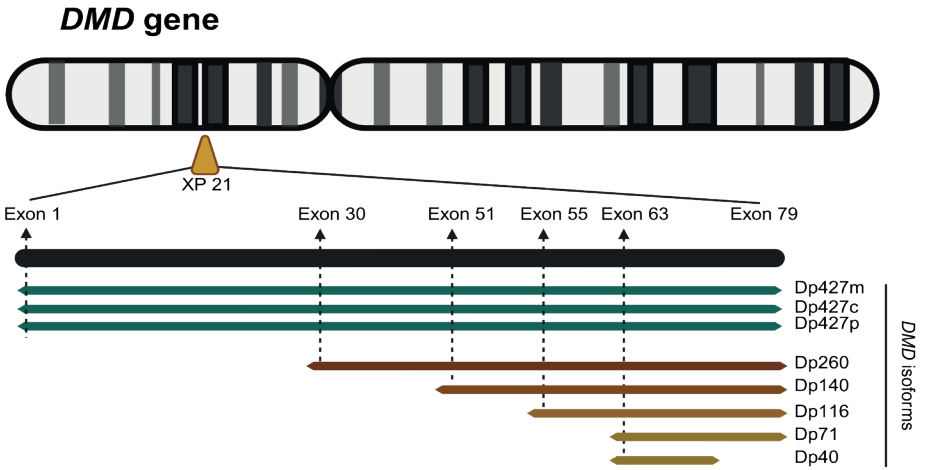


Figure 1. The DMD gene. Graphical representation of the DMD gene and its location on the X-chromosome, the exons and different DMD isoforms with variable length ranging from full-length Dp427 variants to the shortest Dp40 isoform.

In muscle tissue, the full-length isoform Dp427$_m$ and shorter isoform Dp71 are expressed and encode for the dystrophin protein, which is part of the dystrophin-glycoprotein complex (DGC) and serves as an anchor between the cytoskeleton of muscle cells to the extracellular matrix. There are commonalities as well as differences between the dystrophinopathies.

DMD is the most common muscular dystrophy, even though it is still considered a rare disorder with a prevalence of 1 in 5,000 male births. By contrast, BMD is less common with a prevalence of 7-29 in 100,000 males (Mercuri et al., 2019; Salari et al., 2022). On a genetic and molecular level, BMD is caused by mutations in the *DMD* gene, which result in the synthesis of shorter or structurally altered dystrophin protein. These dystrophins retain some functional activity, but often there is a reduced quantity as well as functionality. DMD is typically caused by frameshift mutations such as duplications, deletions or point mutations in the *DMD* gene, resulting in non-functional or complete absence of dystrophin proteins (Duan et al., 2021).

1.1.1 Clinical representation of dystrophinopathies

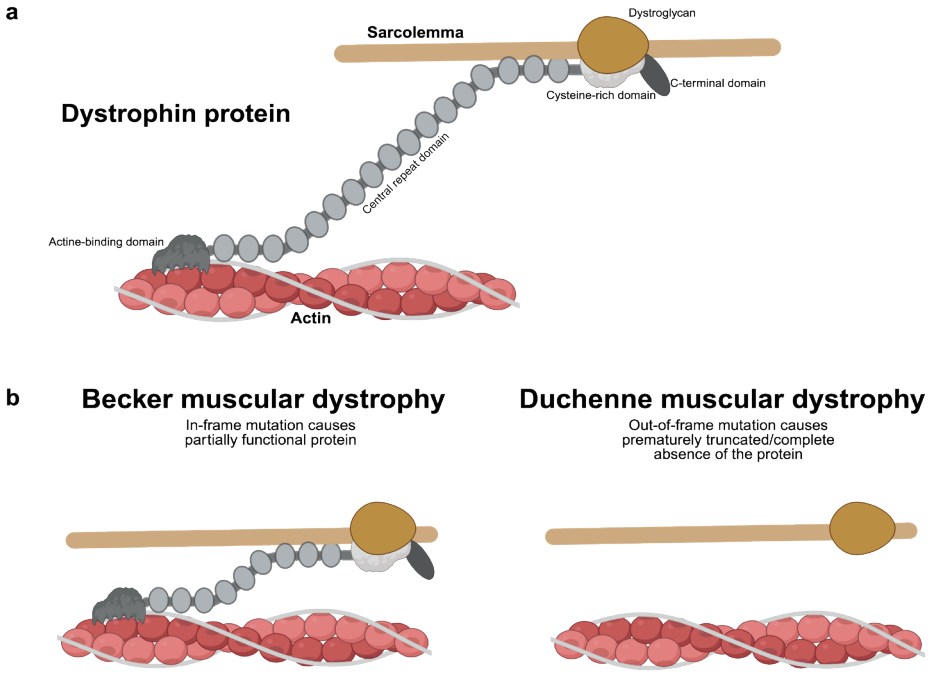


Figure 2. The dystrophin protein in a healthy setting vs. in dystrophinopathies. (a) Dystrophin protein serves as a shock absorber linking actin to the extracellular matrix through the dystroglycan complex in a normal situation. (b) In Becker muscular dystrophy, in-frame mutation causes the production of a partially functional shortened dystrophin protein. Whereas in Duchenne muscular dystrophy, out-of-frame mutation causes prematurely truncated or total loss of the dystrophin protein.

In clinical presentation, dystrophinopathies show the biggest separation. For patients with BMD, symptoms appear later generally speaking, compared to patients with DMD, with an age of diagnosis ranging from early childhood to teenage years or even into adulthood (Mercuri et al., 2019). Whereas for DMD patients, symptoms appear early between 2-3 years of age and the disease progresses more rapidly, leading to loss of ambulation in the early teens and an reduced life expectancy between 20 and 40 years of age due to cardiac failure or respiratory complications (Broomfield et al., 2021; Birnkrant et al., 2018; D'Ambrosio & Mendell, 2023). In general, DMD patients suffer a more severe disease course compared to patients suffering from BMD who may retain walking ability into adulthood. The life expectancy of BMD patients can vary widely, but many individuals live into mid-adulthood or beyond, with a less severe disease course given that some patients never lose ambulation and most patients do not need ventilatory support. However, the most common cause of death is, similar to DMD patients, cardiac failure (Mercuri et al., 2019; Salari et al., 2022).

1.1.2 Current treatment strategies for dystrophinopathies

Although significant research and efforts have been made to develop effective treatments for muscular dystrophies, a cure for dystrophinopathies is still not available. The current treatment strategies for DMD patients focus on early intervention with steroid therapy, physical therapy, cardiac care and later on respiratory support. For BMD patients, management of the disease also includes steroid therapy, physical therapy and cardiac management (Mercuri et al., 2019; Saad, Ficiliano & Angelini, 2023).

Some (potential as well as FDA approved) therapies focus on the cause of the disease and thus restoring the loss of dystrophin. These therapies include gene therapy, antisense oligonucleotide-mediated exon skipping therapy and stop codon readthrough therapy (Saad, Ficiliano & Angelini, 2023; Goemans & Buyse, 2014). Other therapies focus on repairing the damage caused by the loss of the dystrophin protein, also referred to as the secondary pathology. However, to target this secondary pathology (e.g. fibrosis, inflammation, fat infiltration) one must understand the pathomechanisms behind this secondary pathology and find potential therapeutic targets and or biomarkers to longitudinally trace the effect of therapies.

1.2 COGNITIVE INVOLVEMENT IN DYSTROPHINOPATHIES

The *DMD* gene is not exclusively expressed in muscle tissue, where the full-length isoform $Dp427_{m}$ is predominantly expressed. The remaining isoforms, both full-length and shorter ($Dp427_{c\ and\ p}$, Dp260, Dp140, Dp116, Dp71 and Dp40), are expressed in cortical ($Dp427_{c}$, Dp140), hippocampal and cerebellar areas (Dp140), peripheral nerve (Dp116) and ubiquitously throughout the central nervous system (CNS, Dp71 and Dp40) or in specific cell types such as Purkinje cells (Dp427p, Dp71) or tissue type such as the retina (Dp260) (Vaillend et al., 2025). Considering these expression patterns of the *DMD* gene that holds the defect for dystrophinopathies, it may not be surprising that dystrophinopathies are multisystemic disorders. Meaning that they include a cognitive impairment on top of the detrimental muscle wasting.

The prevalence of intellectual disability and behavioral issues (e.g. attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD)) is higher in DMD patients compared to BMD patients where these deficits are less common and individuals may have normal or near-normal intellectual development (Pascual-Morena et al., 2022; Ferrero & Rossi, 2022).

It has been reported that thirty percent of the DMD patients suffer from cognitive, behavioral and/or sensory impairments, which range from severe ASD to obsessive compulsive disorder (OCD), learning difficulties or speech delay (Thangarajh et al., 2019). Furthermore, the average intelligence quotient (IQ) of DMD patients is one standard deviation lower compared to the IQ of the general population (Rosman & Kakulas, 1966; Leibowitz & Dubowitz, 1981; Cotton et al., 2001), whereas patients suffering from BMD have a normal IQ but are still at risk of impairment of the executive functions and development of learning disabilities (Cumbo et al., 2022). An example of such developmental delay that affects DMD patients is the affected language and locomotor areas, which slightly improves over time but causes e.g. speech delay in these boys (Smith et al., 1990; Thangarajh et al., 2019). These neurodevelopmental defects are directly linked to the *DMD* mutation, with an synergistic effect between cognitive impairment and mutations along the *DMD* gene. Meaning that when patients lack more dystrophin isoforms, their chance of being cognitively affected is higher and the patients appear to be more severely affected than patients deficient in only one dystrophin isoform (Chieffo et al., 2015).

1.2.1 Morphological and microstructural brain alterations

Besides the genetic correlation to cognitive impairment, there are also neuropathological findings in terms of morphological and microstructural brain differences. Brain imaging studies involving BMD patients remain limited, likely due to the milder clinical phenotype; consequently, the majority of research in this area has concentrated on DMD patients. However, already in the eighties and nineties, using imaging techniques such as magnetic resonance imaging (MRI), it was shown that cortical atrophy, which was present in 67% of the DMD patients, is associated with minimal ventricular dilatation and white matter abnormalities (Yoshioka et al., 1980; Septien et al., 1991). These findings have repeatedly been reported. In studies by Doorenweerd and colleagues, it was shown that DMD patients have reduced gray matter volume as well as white matter abnormalities and a reduced cerebral perfusion compared to age-matched healthy controls (Doorenweerd et al., 2014; Doorenweerd et al., 2017). Moreover, these studies also linked these structural brain abnormalities to the genetic profile of the patient. They suggest that an additional loss of Dp140 specifically, may lead to abnormal nervous system tract morphology, white matter abnormalities and grey matter reduction, which would explain the reduction in information processing in patients suffering from such a genetic profile (Doorenweerd et al., 2017). Another study confirmed that patients with distal mutations are more frequently reported to show more extensive white matter abnormalities as well as poor neuropsychological performance (Preethish-Kumar 2020).

Besides the effect of the location of the mutation, recent studies also looked into the effect of treatment strategies. While it goes beyond the scope of this thesis to dive deep into these mechanisms, it is important to realize that besides the DMD phenotype, corticosteroid treatment, which is part of the standard of care, has an additional effect on volumetric differences in the brain, impacting the neuropsychological performance of the patients. DMD patients receiving daily treatment have more pronounced brain alterations compared to those treated intermittently (Geuens et al., 2023). A new study describes that both corticosteroid treatment strategy and genotype are linked to variations in subcortical volumes and cortical morphology, although in different manners. Corticosteroid treatment seems to have a more significant association with differences in gray matter characteristics of brain regions related to functional outcomes and neuropsychological functioning (Geuens et al., 2024).

1.2.2 Functional brain differences

These (micro)structural differences in dystrophinopathy patients are suggested to arise from altered brain development and maturation rather than atrophy. However, besides the (micro)structural and morphological differences observed, there have also been studies looking into the functioning of the brain with functional imaging techniques such as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and functional MRI (fMRI) (Angelini & Pinzan, 2019).

Such studies show a hypometabolism of glucose, detected by PET, meaning lower functional activity measurements in boys affected by DMD in the medial temporal structures, cerebellum and the right side of the sensorimotor and lateral temporal cortex (Lee et al., 2002; Bresolin et al., 1994). Moreover, another early study also looked into the brain biochemistry of DMD patients and found altered metabolite concentrations in the left cerebrum as detected with SPECT (Rae et al., 1998). Furthermore, with fMRI, researchers observed a decrease of synchronization of spontaneous activity in the motor cortex. Meaning, a disruption in the normal coordinated firing of neurons in the motor cortex, which may hamper smooth and controlled motor function (Lv et al., 2011). These findings underlie the existence of functional abnormalities found in patients suffering from DMD and might be due to the deficiency of dystrophin in the brain. However, the function and presence of dystrophin in the brain as well as the etiology of the CNS pathology in dystrophinopathies remains poorly understood until this date.

1.2.3 Impact of brain comorbidities and unresolved challenges

Brain comorbidities, though not causative for the early death in DMD patients, can be more impactful on the quality of life than the limitation in mobility caused by the disease according to DMD patient and its caretakers (Hendriksen et al., 2020). It is therefore of great importance to not ignore this aspect of the multifactorial disease and deepen our knowledge on the cognitive impairments in dystrophinopathies, which will aid in better understanding as well as the utilization of proper neuropharmacology in this patient group (Hendriksen et al., 2020).

Given that progressive muscle wasting is the primary cause of mortality in patients with Duchenne muscular dystrophy (DMD), research has predominantly concentrated on this aspect of the disease. However, over the last decade, more research has focused on the role of dystrophin in the brain as well as the neurobehavioral and biological implications of dystrophinopathies. Increasing our knowledge on the expression of the *DMD* gene throughout the central nervous system is of great importance for better understanding the pathomechanisms in the patients with their accompanied neurobehavioral challenges, the effects of treatment strategies on these defects as well as that it will aid in finding neuropharmacological targets and or support strategies.

1.3 MUSCLE WASTING IN DYSTROPHINOPATHIES

1.3.1 Muscle degeneration and regeneration

In order to understand the muscle wasting in dystrophinopathies, let's consider the healthy skeletal muscle first. Healthy skeletal muscle consists of multiple cell types, all contributing to well-functioning (e.g. contracting, regenerating) muscle. The main cell type is the myofiber, a multinucleated cell type that has three main types based on their myosin heavy chain isoforms: slow oxidative (type I), fast oxidative (type IIa) and fast glycolytic (type IIx) (Staron, 1997; Scott, Stevens & Binder-Macleod, 2001). The different fiber types are characterized by their metabolic and contractile properties ranging from slow contractile and fatigue-resistant to fast contractile and not fatigue-resistant (Scott, Stevens & Binder-Macleod, 2001; Barany, 1967). Originally, there was only a division between fast and slow fiber types. However, intermediate fiber types have also been identified with histological staining, leading to a total of seven fiber types (Scott, Stevens & Binder-Macleod, 2001). Nevertheless, many studies focus on the identification of the original three fiber types.

Upon muscle damage, which also happens in healthy skeletal muscle when an individual trains their muscles through exercise or upon traumatic injury, the muscle will attempt to repair the damage made through cycles of degeneration and regeneration. This is where the mononucleated cell types, resident in the skeletal muscle, come into play. First, various innate immune cells such as neutrophils and macrophages are recruited to the injured site. The neutrophils release pro-inflammatory cytokines, proteases and reactive oxygen species (ROS) and both, the M1 macrophages and neutrophils, help remove the dead cells and debris through phagocytosis (Ziemkiewicz et al., 2021; Gehlert & Jacko, 2019). After clearing the debris, the M2 macrophages exhibit a pro-regenerative phenotype and secrete cytokines as well as growth factors like IL-10, TGF-β and IGF-1 to enhance muscle repair, satellite cell activation and in turn myogenesis (Ziemkiewicz et al., 2021). Next to these macrophages, there is another non-myogenic cell population important for myofiber regeneration as well as rebuilding the extracellular matrix, the fibro-adipogenic progenitor cells (FAPs). In response to injury, FAPs will proliferate and also secrete trophic factors to stimulate the myogenic activity of the satellite cells. Satellite cells, known as the stem cells of the muscle, proliferate and differentiate into myocytes that become myofibers, completing the successful regeneration.

Satellite cells, known as the stem cells of the muscle, proliferate and differentiate into myocytes that become myofibers, completing the successful regeneration. Satellite cells themselves can also directly fuse into myofibers (Hao et al., 2022; Axelrod et al., 2021; Liu et al., 2018; Mázala et al., 2020; Yin, Price & Rudnicki, 2013). Other cell types such as the including endothelial cells, dendritic cells, adipocytes, as well as components of the neuromuscular and myotendinous junctions, are also present within healthy and diseased skeletal muscle where they perform important physiological functions. Even though their roles are generally not the primary focus in studies addressing the degeneration and regeneration of muscle fibers it is good to keep in mind these cells all play their part in the skeletal muscle environment.

1.3.2 Chronic injury in dystrophinopathies

In patients suffering from dystrophinopathy, the regenerative capacity of the myofibers becomes exhausted over time after extensive regeneration. The clinical manifestations of the dystrophinopathies, with e.g. loss of ambulation leading to wheelchair dependency, cardiomyopathy and respiratory insufficiency are caused by pathophysiological defects. At the basis there is a genetic defect leading to a loss of the dystrophin protein that was previously described which has ample secondary pathological consequences that affect the skeletal and cardiac muscle. The absence of (functional) dystrophin in dystrophinopathies leads to a loss of muscle fiber stability as the protein acts as a linker between the intracellular actin cytoskeleton and extracellular matrix. Consequently, muscle fibers are prone to contraction-induced damage, which leads to chronic inflammation, cycles of impaired regeneration and degeneration, and eventually, increased deposition of fat and fibrosis in the skeletal muscle tissue (Duan et al., 2021; Molina, Fabre & Dumont, 2021; Parker & Hamrick, 2021).

Chronic satellite cell activation to repair injury to the muscle fibers, leads to senescence of the satellite cells. The impossibility to complete muscle fiber repair leads to a constant secretion of pro-inflammatory cytokines. In this environment the FAPs as well as the macrophages continue to proliferate and differentiate. An overactive, or prolonged, macrophage response contributes to muscle degeneration by sustaining this chronic inflammatory profile, excessive production of ROS and inability to switch to a pro-regenerative M2 macrophage phenotype (Hernandez-Torres et al., 2024).

FAPs, in turn, continue to proliferate and differentiate into adipocytes and/or fibroblasts, leading to increased fat and fibrosis depositions or fibrofatty replacement in the tissue. This leads to the complete substitution of muscle with adipose tissue, which is a prognostic biomarker in DMD and other muscular dystrophies (Molina, Fabre & Dumont, 2021; Parker & Hamrick, 2021).

1.3.3 Known cell types and unresolved mechanisms

At the histological level, dystrophic muscle presents various observations, e.g. inflammation, increased fibrosis, adipocyte infiltration, varied muscle fiber size, centralized nuclei and a loss of polygonal shape of muscle fibers. Such microscopic alterations can be observed at the histological level. However, technologies such as DTI or high field MRI could potentially provide metrics for this in vivo.

To understand the molecular changes associated with histological alterations, omic studies have been performed in patients' derived muscle biopsies and in mouse models (Haslett et al., 2002; Pescatori et al., 2007; Sterrenburg et al., 2004; Van Putten et al., 2012). Gene expression studies especially contributed to identify pathways altered in dystrophic muscles and how the lack of the coordinated action of cells and pathways primes the skeletal muscle tissue to the formation of tissue lesions such as fibrosis and adipose tissue deposition (Dadgar et al., 2014).

However, the exact mechanisms by which the muscle is replaced by fibrotic or adipose tissue, including the molecular drivers, underlying genes and pathways remain poorly understood. In order to find potential therapeutic targets and develop successful therapies targeting the secondary pathology, a better understanding of the basic pathomechanisms of dystrophinopathies is needed.

1.3.4 Biomarker discovery: why and how?

As described before, biomarkers are crucial in studying neuromuscular disorders for multiple reasons: 1) early diagnosis and detection, 2) (non-invasive) monitoring of disease progression, 3) treatment response assessment, 4) design of (personalized) medicine, 5) evaluating clinical trial efficacy and 6) understanding disease mechanisms.

The National Institutes of Health (NIH) defined biomarkers in 2001 as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other healthcare intervention' (Biomarkers Definitions Working Group, 2001). One could thus think of biomarkers as tools that can anticipate, predict and monitor disease progression and/or treatment response. It has been shown that the use of selected biomarkers in clinical trials significantly increases the success of the trial, which underlines the need for good biomarkers (Davis et al., 2020). Moreover, biomarkers aid in evaluating the efficacy of the experimental treatment and can guide optimal dosing, duration and stratification of the patient population for a new drug. Measuring biomarkers can be done at different levels, ranging from DNA to protein and from RNA to magnetic MRI parameters. Measuring biomarkers is thus also performed using different inputs: body fluids (e.g. urine, blood), tissue (e.g. biopsy, cells, skin) or indirectly and less invasive using imaging technologies. Regardless of the method, recent technological advancements (transcriptomics, metabolomics, proteomics, imaging techniques) allow for more biomarker discovery than ever before.

One may argue that the process of biomarker discovery is similar to, or maybe is part of, better understanding disease mechanisms, which may lead to identifying potential therapeutic targets and/or biomarkers. This thesis stresses that, even though the focus on treatment development is understandable, increasing our knowledge on the pathological mechanisms in neuromuscular diseases is still very much needed in order to identify and improve treatment strategies.

By better understanding the basic mechanisms, and identifying molecular drivers of histopathological tissue changes, one may be able to target the so-called secondary pathology e.g. fibrosis, necrosis, inflammation and fat infiltration.

1.4 A NEW APPROACH TO BETTER UNDERSTAND THE PATHOMECHANISMS IN DYSTROPHINOPATHIES

In recent years, new methods able to capture molecular signatures in individual cells and tissue sections became available. This thesis focuses on the use and application of single-cell RNA sequencing (scRNA-seq) as well as spatial transcriptomics to aid in better understanding the pathomechanisms in dystrophinopathies (Chapter 2, 4 and 5).

These technologies are part of the 'omics' tools and era. The era of omics began with the mapping of the human genome with the Human Genome Project (HGP), which aimed to map the entire human genome identifying all the genes and describe their sequence. This project began in 1990 and was completed in 2003, marking a monumental achievement in biology and genomics. It laid the foundation for studying entire biological networks and not only genes. Moreover, the HGP accelerated with the development of new technologies for high-throughput data generation and analysis (National Academy of Science, 1988; Human Genome Sequencing Consortium, 2004). It started off with the now-called 'first-generation sequencing' approach that relied on labor intensive gel electrophoresis (Smith et al., 1986). Thanks to the combined efforts of researchers and biotech companies, technologies rapidly improved in costs, speed, throughput and accuracy the first automated versions of these first-generation sequencing technologies were developed.

Fast forward to 2004, when Dr. Jonathan Rothberg with 454 Life Sciences developed the first commercially available 'next generation sequencing' (NGS) platform, which was based on pyrosequencing and able to sequence DNA much faster than the traditional Sanger method. In 2006, Illumina, founded by Dr. Shankar Ramaswamy, released another sequencing technology that was based on sequencing by synthesis (SBS) and reduced the costs and time significantly compared to earlier methods. Many more companies such as Applied Biosystems and PacBio contributed to this transformation of genomics and allowed to immensely reduce the costs and time required for DNA sequencing. From this, and in parallel, the field of 'omics' expanded from genomics (studying the genome) to include transcriptomics (studying RNA molecules), proteomics (studying proteins), metabolomics (studying metabolites) and many more. The discovery of biomarkers can, as stated before, be done at any of these levels (e.g. DNA, RNA).

This thesis focuses on transcriptomics where the RNA molecules, including messenger RNA and non-coding RNA that expressed in a tissue at a given time are studied. Within the field of transcriptomics, we see an evolution of studying the RNA with qPCRs, microarrays towards improving the RNA sequencing technique from bulk RNA sequencing towards, now, high resolution spatial transcriptomics (Figure 3).

Bulk approaches such as RNA-seq allow to detect the expression of genes in a tissue or sample (Figure 3a). Recently developed single cell technologies such as scRNA-seq, allow to map gene expression signatures to individual cells, therefore providing more granular information (Figure 3b). While scRNA-seq allows for more detailed information, the spatial context of the individual cells and the microenvironment or cross-cell connections is lost during isolation of cells. While this is not a limitation for liquid tissues such as blood, this is a limitation for solid tissues. Such a limitation is even larger for skeletal muscle tissue, due to the multi-nucleated nature of muscle fibers. Spatial transcriptomics allows to overcome such limitations by allowing the analysis of gene expression in single tissue sections. The tissue of interest is sectioned after which this section is stained and imaged. Hereafter, the RNA is retracted from that tissue section and prepped for sequencing. Hereafter, the image and thus spatial context can be aligned to the sequencing data, revealing which genes were expressed where in the tissue (Figure 3c).

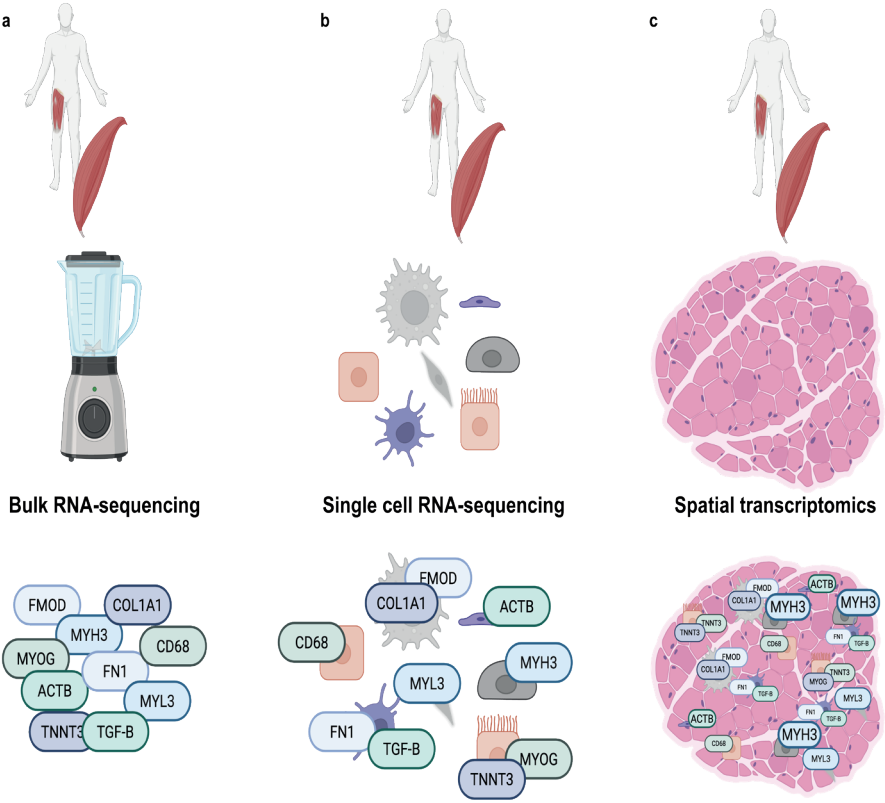


Figure 3. Gene expression methods: from bulk RNA-seq to spatial transcriptomics. (a) Bulk RNA-seq can be seen as taking a biopsy, processing it in the blender and getting back which genes were expressed in that piece of tissue. (b) Whereas scRNA-seq builds on this and you first isolate the single cells from the tissue after which you sequence them and get back which genes were expressed in the tissue. (c) Finally, spatial transcriptomics processes (images and sequence) a single section of the tissue and reveals which genes are expressed where in the tissue spatially.

1.5 SCOPE AND OUTLINE OF THE THESIS

The research presented in this thesis addresses various previously unanswered challenges in the understanding and combating of various NMDs, with a special focus on dystrophinopathies. Throughout this thesis, single cell and spatial gene expression technologies are used to address gaps in the current literature.

In **Chapter 2**, the expression patterns of the *Dmd* gene and its isoforms in the (mostly murine) central nervous system are being studied with the use of scRNA-seq. Mapping the expression of *Dmd* in different brain areas, cell types, as well as over time and the consequences of a lack of specific *Dmd* isoforms in DMD mouse models was explored further. **Chapter 3**, dives into the potential use and validation of using DT-MRI metrics as a noninvasive biomarker for muscular dystrophies, especially BMD, where the DT-MRI metrics align with histological measures. Hereafter, in **Chapter 4**, we explore the use of a new technology, spatial transcriptomics, on dystrophic skeletal muscle for the first time to reveal molecular markers of histopathological tissue changes in DMD mouse models. This Chapter reveals the potential of the technology for unbiased marker detection and deepening our knowledge on the basic pathomechanisms in NMDs. Finally, **Chapter 5** encompasses work on spatial transcriptomic experiments and analysis of dystrophinopathy patients. We dive further into specific tissue alterations such as fibrofatty infiltration, as well as the interaction of cells in the diseased tissue microenvironment by using scRNA-seq data. **Chapter 6** summarizes and discusses the results presented in the Chapters mentioned above.

REFERENCES (ALPHABETICAL ORDER)

Aartsma-Rus, A., Van Deutekom, J. C., Fokkema, I. F., Van Ommen, G. J. B., & Den Dunnen, J. T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine, 34(2), 135-144.

Angelini, C., & Pinzan, E. (2019). Advances in imaging of brain abnormalities in neuromuscular disease. Therapeutic advances in neurological disorders, 12, 1756286419845567.

Axelrod, C. L., Fealy, C. E., Erickson, M. L., Davuluri, G., Fujioka, H., Dantas, W. S., ... & Kirwan, J. P. (2021). Lipids activate skeletal muscle mitochondrial fission and quality control networks to induce insulin resistance in humans. Metabolism, 121, 154803.

Barany, M. (1967). ATPase activity of myosin correlated with speed of muscle shortening. Journal of General Physiology, 50(6), 197-218
.
Biomarkers Definitions Working Group: Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001;69:89–95.

Birnkrant, D. J., Bushby, K., Bann, C. M., Apkon, S. D., Blackwell, A., Brumbaugh, D., ... & Weber, D. R. (2018). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management. The Lancet Neurology, 17(3), 251-267.

Bresolin, N., Castelli, E., Comi, G. P., Felisari, G., Bardoni, A., Perani, D., ... & Scarlato, G. (1994). Cognitive impairment in Duchenne muscular dystrophy. Neuromuscular Disorders, 4(4), 359-369.

Broomfield, J., Hill, M., Guglieri, M., Crowther, M., & Abrams, K. (2021). Life expectancy in Duchenne muscular dystrophy: reproduced individual patient data meta-analysis. Neurology, 97(23), e2304-e2314.

Byers, T. J., Lidov, H. G., & Kunkel, L. M. (1993). An alternative dystrophin transcript specific to peripheral nerve. Nature genetics, 4(1), 77-81.

Chelly, J., & Hamard, G. (1990). Dystrophin gene transcribed from different promoters in neuronal and glial cells. Nature, 344(6261).

Chieffo, D., Brogna, C., Berardinelli, A., D'Angelo, G., Mallardi, M., D'Amico, A., ... & Pane, M. (2015). Early neurodevelopmental findings predict school age cognitive abilities in Duchenne muscular dystrophy: a longitudinal study. PLoS One, 10(8), e0133214.

Cotton, S., Voudouris, N. J., & Greenwood, K. M. (2001). Intelligence and Duchenne muscular dystrophy: full-scale, verbal, and performance intelligence quotients. Developmental medicine and child neurology, 43(7), 497-501.

Cumbo, F., Tosi, M., Catteruccia, M., Diodato, D., Nicita, F., Capitello, T. G., ... & D'Amico, A. (2022). Neuropsychological and behavioral profile in a cohort of Becker muscular dystrophy pediatric patients. Neuromuscular Disorders, 32(9), 736-742.

Dadgar, S., Wang, Z., Johnston, H., Kesari, A., Nagaraju, K., Chen, Y. W., ... & Hoffman, E. P. (2014). Asynchronous remodeling is a driver of failed regeneration in Duchenne muscular dystrophy. Journal of Cell Biology, 207(1), 139-158.

D'Ambrosio, E. S., & Mendell, J. R. (2023). Evolving therapeutic options for the treatment of duchenne muscular dystrophy. Neurotherapeutics, 20(6), 1669-1681.

Davis, K. D., Aghaeepour, N., Ahn, A. H., Angst, M. S., Borsook, D., Brenton, A., ... & Pelleymounter, M. A. (2020). Discovery and validation of biomarkers to aid the development of safe and effective pain therapeutics: challenges and opportunities. Nature Reviews Neurology, 16(7), 381-400.

Doorenweerd, N., Mahfouz, A., van Putten, M., Kaliyaperumal, R., t'Hoen, P. A., Hendriksen, J. G., ... & Lelieveldt, B. P. (2017). Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. Scientific reports, 7(1), 12575.

Doorenweerd, N., Straathof, C. S., Dumas, E. M., Spitali, P., Ginjaar, I. B., Wokke, B. H., ... & Kan, H. E. (2014). Reduced cerebral gray matter and altered white matter in boys with Duchenne muscular dystrophy. Annals of Neurology, 76(3), 403-411.

Doorenweerd, N., Dumas, E. M., Ghariq, E., Schmid, S., Straathof, C. S., Roest, A. A., ... & Kan, H. E. (2017). Decreased cerebral perfusion in Duchenne muscular dystrophy patients. Neuromuscular Disorders, 27(1), 29-37.

D'Souza, V. N., Man, N. T., Morris, G. E., Karges, W., Pillers, D. A. M., & Ray, P. N. (1995). A novel dystrophin isoform is required for normal retinal electrophysiology. Human molecular genetics, 4(5), 837-842.

Duan, D., Goemans, N., Takeda, S. I., Mercuri, E., & Aartsma-Rus, A. (2021). Duchenne muscular dystrophy. Nature reviews disease primers, 7(1), 13.

Ferrero, A., & Rossi, M. (2022). Cognitive profile and neuropsychiatric disorders in Becker muscular dystrophy: a systematic review of literature. Neuroscience & Biobehavioral Reviews, 137, 104648.

Gehlert, S., & Jacko, D. (2019). The Role of the Immune System in Response to Muscle Damage. German Journal of Sports Medicine/Deutsche Zeitschrift fur Sportmedizin, 70(10).

Geuens, S., Van Dessel, J., Govaarts, R., Ikelaar, N. A., Meijer, O. C., Kan, H. E., ... & De Waele, L. (2023). Comparison of two corticosteroid regimens on brain volumetrics in patients with Duchenne muscular dystrophy. Annals of Clinical and Translational Neurology, 10(12), 2324-2333.

Geuens, S., Goemans, N., Lemiere, J., Doorenweerd, N., & De Waele, L. (2024). Duchenne Muscular Dystrophy-Associated Neurobehavioral Difficulties: Insights from Clinical Practice. Journal of neuromuscular diseases, 11(4), 791-799.

Goemans, N., & Buyse, G. (2014). Current treatment and management of dystrophinopathies. Current treatment options in neurology, 16, 1-13.

Górecki, D. C., Monaco, A. P., Derry, J. M., Walker, A. P., Barnard, E. A., & Barnard, P. J. (1992). Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. Human molecular genetics, 1(7), 505-510.

Hao, D., Becker, N., Mückter, E., Müller, A., Pishnamaz, M., Bollheimer, L. C., ... & Nourbakhsh, M. (2022). In Vitro Model of Human Skeletal Muscle Tissue for the Study of Resident Macrophages and Stem Cells. Biology, 11(6), 936.

Haslett, J. N., Sanoudou, D., Kho, A. T., Bennett, R. R., Greenberg, S. A., Kohane, I. S., ... & Kunkel, L. M. (2002). Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. Proceedings of the National Academy of Sciences, 99(23), 15000-15005.

Hendriksen, J. G., Thangarajh, M., Kan, H. E., Muntoni, F., Aoki, Y., Collin, P., ... & Vroom, E. (2020). 249th ENMC International Workshop: The role of brain dystrophin in muscular dystrophy: Implications for clinical care and translational research, Hoofddorp, The Netherlands, November 29th–December 1st 2019. Neuromuscular Disorders, 30(9), 782-794.

Hernandez-Torres, F., Matias-Valiente, L., Alzas-Gomez, V., & Aranega, A. E. (2024). Macrophages in the Context of Muscle Regeneration and Duchenne Muscular Dystrophy. International Journal of Molecular Sciences, 25(19), 10393.

Hugnot, J. P., Gilgenkrantz, H., Vincent, N., Chafey, P., Morris, G. E., Monaco, A. P., ... & Kahn, A. (1992). Distal transcript of the dystrophin gene initiated from an alternative first exon and encoding a 75-kDa protein widely distributed in nonmuscle tissues. Proceedings of the National Academy of Sciences, 89(16), 7506-7510.

Human Genome Sequencing Consortium: Finishing the euchromatic sequence of the human genome. Nature. 2004, 431: 931-945. 10.1038/nature03001.

Laing, N. G. (2012). Genetics of neuromuscular disorders. Critical reviews in clinical laboratory sciences, 49(2), 33-48.
Lee, J. S., Pfund, Z., Juhász, C., Behen, M. E., Muzik, O., Chugani, D. C., ... & Chugani, H. T. (2002). Altered regional brain glucose metabolism in Duchenne muscular dystrophy: a pet study. Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine, 26(4), 506-512.

Leibowitz, D., & Dubowitz, V. (1981). Intellect and behaviour in Duchenne muscular dystrophy. Developmental Medicine & Child Neurology, 23(6), 577-590.

Lidov, H. G., Selig, S., & Kunkel, L. M. (1995). Dp140: a novel 140 kDa CNS transcript from the dystrophin locus. Human molecular genetics, 4(3), 329-335.

Liu, G., Liao, C., Chen, X., Xu, Y., Tan, J., Han, F., & Ye, X. (2018). Identification and characterization of skeletal muscle stem cells from human orbicularis oculi muscle. Tissue Engineering Part C: Methods, 24(8), 486-493.

Lv, S. Y., Zou, Q. H., Cui, J. L., Zhao, N., Hu, J., Long, X. Y., ... & Zang, Y. F. (2011). Decreased gray matter concentration and local synchronization of spontaneous activity in the motor cortex in Duchenne muscular dystrophy. American journal of neuroradiology, 32(11), 2196-2200.

Mázala, D. A., Novak, J. S., Hogarth, M. W., Nearing, M., Adusumalli, P., Tully, C. B., ... & Partridge, T. A. (2020). TGF-β–driven muscle degeneration and failed regeneration underlie disease onset in a DMD mouse model. JCI insight, 5(6), e135703.

Mercuri, E., Bönnemann, C. G., & Muntoni, F. (2019). Muscular dystrophies. The Lancet, 394(10213), 2025-2038.
Molina, T., Fabre, P., & Dumont, N. A. (2021). Fibro-adipogenic progenitors in skeletal muscle homeostasis, regeneration and diseases. Open biology, 11(12), 210110.

National Academy of Science: Report of the Committee on Mapping and Sequencing the Human Genome. 1988, Washington DC: National Academy Press

Parker, E., & Hamrick, M. W. (2021). Role of fibro-adipogenic progenitor cells in muscle atrophy and musculoskeletal diseases. Current opinion in pharmacology, 58, 1-7.

Pascual-Morena, C., Cavero-Redondo, I., Reina-Gutiérrez, S., Saz-Lara, A., López-Gil, J. F., & Martínez-Vizcaíno, V. (2022). Prevalence of neuropsychiatric disorders in Duchenne and Becker muscular dystrophies: a systematic review and meta-analysis. Archives of Physical Medicine and Rehabilitation, 103(12), 2444-2453.

Pescatori, M., Broccolini, A., Minetti, C., Bertini, E., Bruno, C., D'amico, A., ... & Ricci, E. (2007). Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression. The FASEB Journal, 21(4), 1210-1226.

Preethish-Kumar, V., Shah, A., Kumar, M., Ingalhalikar, M., Polavarapu, K., Afsar, M., ... & Saini, J. (2020). In vivo evaluation of white matter abnormalities in children with Duchenne muscular dystrophy using DTI. American Journal of Neuroradiology, 41(7), 1271-1278.

Van Putten, M., Hulsker, M., Nadarajah, V. D., van Heiningen, S. H., van Huizen, E., van Iterson, M., ... & Aartsma-Rus, A. (2012). The effects of low levels of dystrophin on mouse muscle function and pathology. PloS one, 7(2), e31937.

Rae, C., Scott, R. B., Thompson, C. H., Dixon, R. M., Dumughn, I., Kemp, G. J., ... & Radda, G. K. (1998). Brain biochemistry in Duchenne muscular dystrophy: a 1H magnetic resonance and neuropsychological study. Journal of the neurological sciences, 160(2), 148-157.

ROSMAN, N. P., & KAKULAS, B. A. (1966). Mental deficiency associated with muscular dystrophy: a neuropathological study. Brain, 89(4), 769-788.

Saad, F. A., Siciliano, G., & Angelini, C. (2023). Advances in dystrophinopathy diagnosis and therapy. Biomolecules, 13(9), 1319.

Salari, N., Fatahi, B., Valipour, E., Kazeminia, M., Fatahian, R., Kiaei, A., ... & Mohammadi, M. (2022). Global prevalence of Duchenne and Becker muscular dystrophy: a systematic review and meta-analysis. Journal of orthopaedic surgery and research, 17(1), 96.

Scott, W., Stevens, J., & Binder–Macleod, S. A. (2001). Human skeletal muscle fiber type classifications. Physical therapy, 81(11), 1810-1816.

Septien, L., Gras, P., Borsotti, J. P., Giroud, M., Nivelon, J. L., & Dumas, R. (1991). Mental development in Duchenne muscular dystrophy. Correlation of data of the brain scanner. Pediatrie, 46(12), 817-819.

Smith, R. A., Sibert, J. R., & Harper, P. S. (1990). Early development of boys with Duchenne muscular dystrophy. Developmental Medicine & Child Neurology, 32(6), 519-527.

Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell CR, Heiner C, Kent SBH, Hood LE: Fluorescence detection in automated DNA sequence analysis. Nature. 1986, 321: 674-679. 10.1038/321674a0.

Staron, R. S. (1997). Human skeletal muscle fiber types: delineation, development, and distribution. Canadian Journal of Applied Physiology, 22(4), 307-327.

Sterrenburg, E., Turk, R., AC't Hoen, P., van Deutekom, J. C., Boer, J. M., van Ommen, G. J. B., & den Dunnen, J. T. (2004). Large-scale gene expression analysis of human skeletal myoblast differentiation. Neuromuscular Disorders, 14(8-9), 507-518.

Thangarajh, M., Kaat, A. J., Bibat, G., Mansour, J., Summerton, K., Gioia, A., ... & Wagner, K. R. (2019). The NIH Toolbox for cognitive surveillance in Duchenne muscular dystrophy. Annals of clinical and translational neurology, 6(9), 1696-1706.

Tinsley, J. M., Blake, D. J., & Davies, K. E. (1993). Apo-dystrophin-3: a 2.2 kb transcript from the DMD locus encoding the dystrophin glycoprotein binding site. Human molecular genetics, 2(5), 521-524.

Vaillend, C., Aoki, Y., Mercuri, E., Hendriksen, J., Tetorou, K., Goyenvalle, A., & Muntoni, F. (2025). Duchenne muscular dystrophy: recent insights in brain related comorbidities. Nature Communications, 16(1), 1298.

Yin, H., Price, F., & Rudnicki, M. A. (2013). Satellite cells and the muscle stem cell niche. Physiological reviews, 93(1), 23-67.

Yoshioka M, Okuno T, Honda Y, et al. Central nervous system involvement in progressive muscular dystrophy. Arch Dis Child 1980; 55: 589–594.

Ziemkiewicz, N., Hilliard, G., Pullen, N. A., & Garg, K. (2021). The role of innate and adaptive immune cells in skeletal muscle regeneration. International journal of molecular sciences, 22(6), 3265.