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Dipartimento di Neuroscienze

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TESI DI LAUREA

"A genome-wide association study of age at loss of ambulation in Duchenne muscular dystrophy"

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ABSTRACT

Duchenne muscular Dystrophy is a severe neuromuscular disorder affecting children. A major obstacle to the development of effective therapies has been its phenotypic variability, which is partly due to the patients' genetic background. Variants in five loci (i.e. genetic modifiers) have been associated with variability in DMD severity: *SPP1*, *LTBP4*, *CD40*, *ACTN3*, and *THBS1*. The purpose of this study is to perform a genome-wide association study (GWAS) to search for novel DMD modifier loci, using age at Loss of Ambulation as a primary indicator of disease severity, and genotyping a large cohort of DMD patients followed at specialized Centres in Italy. We identify association signals in chromosomes 1. Neighbouring genes represent putative modifiers to be subjected to functional studies.

INTRODUCTION

Duchenne muscular dystrophy is a rare neuromuscular disease affecting children. It is the most severe form of dystrophinopathy. It is a lethal X-linked recessive disorder caused by mutations in *DMD*, the gene encoding dystrophin. Prevalence of DMD is 15.9/100000 males in the USA (1) and has an incidence of one in approximately 5000 live male births (2). It is characterized by a progressive loss of muscle and deterioration of skeletal muscle, cardiac, and lung function; therefore, it may be considered a multisystemic disease.

Role of dystrophin and pathogenesis

Skeletal muscle tissue consists of muscle fibres (which are syncitia of fused muscle cells), clustered into bundles. The entire muscle is surrounded by the epimysium (connective tissue). The underlying layer is called perimysium, which envelops bundles of fibres. In turn, a single muscle fibre is covered by endomysium. A single muscle fibre is composed of myofibrils, then the set myofilaments form the sarcomere, the muscle functional unit. The muscle cell membrane is called sarcolemma, and it is protected from contraction-induced injury by specific protein complexes which link the sarcomere to the extracellular matrix (3). The

malfunction or lack of any of these proteins causes damage to the membrane and interferes with the contraction process. One of the most essential proteins for the complex is dystrophin. It contributes to form the DGC (dystrophin-glycoprotein complex) which takes different roles in stability and contraction. (4) The DGC includes dystrophin, dystroglycan and sarcoglycans. The four functional domains of dystrophin are the amino-terminus, the rod region, the carboxyl-terminus and the extreme carboxyl-terminal region (5). To regulate muscle contraction, the first function of dystrophin is to handle Ca2+. On the other hand, it protects sarcolemma from contraction-induced oxidative stress. In Duchenne muscular dystrophy, mutations lead to a lack of this protein provoking a destabilization of DCG. The binding complex is compromised causing muscle degeneration. Mutation of dystrophin prevents distribution of the mechanical forces during muscle contraction. Furthermore, stress production is increased in dystrophin muscle preventing proper function. (6) Finally, an altered reparation results in fibrosis and fat replacement. (7)

A gene localized in Xp21 encodes dystrophin. The dystrophin gene is the largest gene known in human beings, increasing the risk of random mutational events. The coding sequence is about 1%, i.e. 86 exons. Since there exist various isoforms, mutations guide to several different affected areas. Moreover, different promoters drive expressions in different areas, generated through splicing. Long isoforms of dystrophin are essential for the muscle. Instead of short isoforms of dystrophin rising from different promoters. These promoters exploit only one exon generating four isoforms: Dp260, Dp140, Dp116, Dp71. Dp260 is expressed in the retina, whereas Dp140 shows itself in the brain, retina, and kidney tissues. Dp71 affects the brain, retina, kidney and liver. Finally, Dp116 influences peripheral nerves (8). Most mutations are hereditary (2/3), the rest is “de novo”. The most frequent mutations are deletion (65%), duplications (9%), and small mutations (25%, e.g. point mutations) and atypical mutations (<1%). Deletions can be at any point in the gene, but are more frequently located in the central part of the gene (exon 45-55) or near the 5’ end (mutational “hotspots"). About 75% of Duchenne Muscular Dystrophy mutations are Copy Number Variations (CNV). There is no simple direct relationship between the mutation extent and the clinical

effect. For example, some patients are paucisymptomatic or with only an elevation of CPK, provided that presenting “in frame” deletions of some exons (for example 32-44, 48-51, 48-53). Even large deletions may be compatible with a relatively mild clinical picture, as seen in Becker Muscular Dystrophy, the milder form of dystrophinopathy. Clinical severity is connected with the disruption of the reading frame (also known as “out of frame”), leading to the production of a truncated protein that rapidly degrades in the muscle, or generation of a premature stop codon. (9) The lack of dystrophin in DMD is caused by the production of a truncated non-functional dystrophin protein, derived from frame-shift mutations, shifting the translational open reading frame (ORF) of triplet codons of amino acids. On the other hand, BMD patients predict a semi functional protein, deriving from mutations that maintain the translational ORF for amino acids. Although these criteria, exceptions exist. (10) “Out of frame” patients could develop DMD, BMD, or an intermediate phenotype as a result of an exon skipping event. (3) (8)

Diagnosis

The purpose of a correct diagnosis is to start as soon as possible a targeted therapy and a multidisciplinary follow-up. Typically, the first step in diagnosis of DMD is the observation of muscle weakness; the second most frequent red flag is the occasional finding CPK elevation (10 to 20 times increased levels) or an increase in transaminases. These symptoms call for a specific neuromuscular examination (11).

When patients are three or four years old, parents start reporting frequent falls or delays in psychomotor development. During the neurological examination, specific protocols are adopted to test some skills such as the ability to get up from the floor and climb the stairs. Toe walking and pseudo-hypertrophic calves represent two suggestive signs. However, Gower’s manoeuvre is considered the most typical sign of DMD. The patient implements compensation manoeuvres to alleviate difficulties in getting up: first, they put a hand on the ground, raising their hip while facing the floor, “climbing” up by leaning both their hands alternately on their legs and then bringing the trunk into the upright posture. (12) After a clinical

evaluation and a finding of highly elevated CK levels, genetic tests and a muscle biopsy should be considered, especially when the family history is unknown. (11)

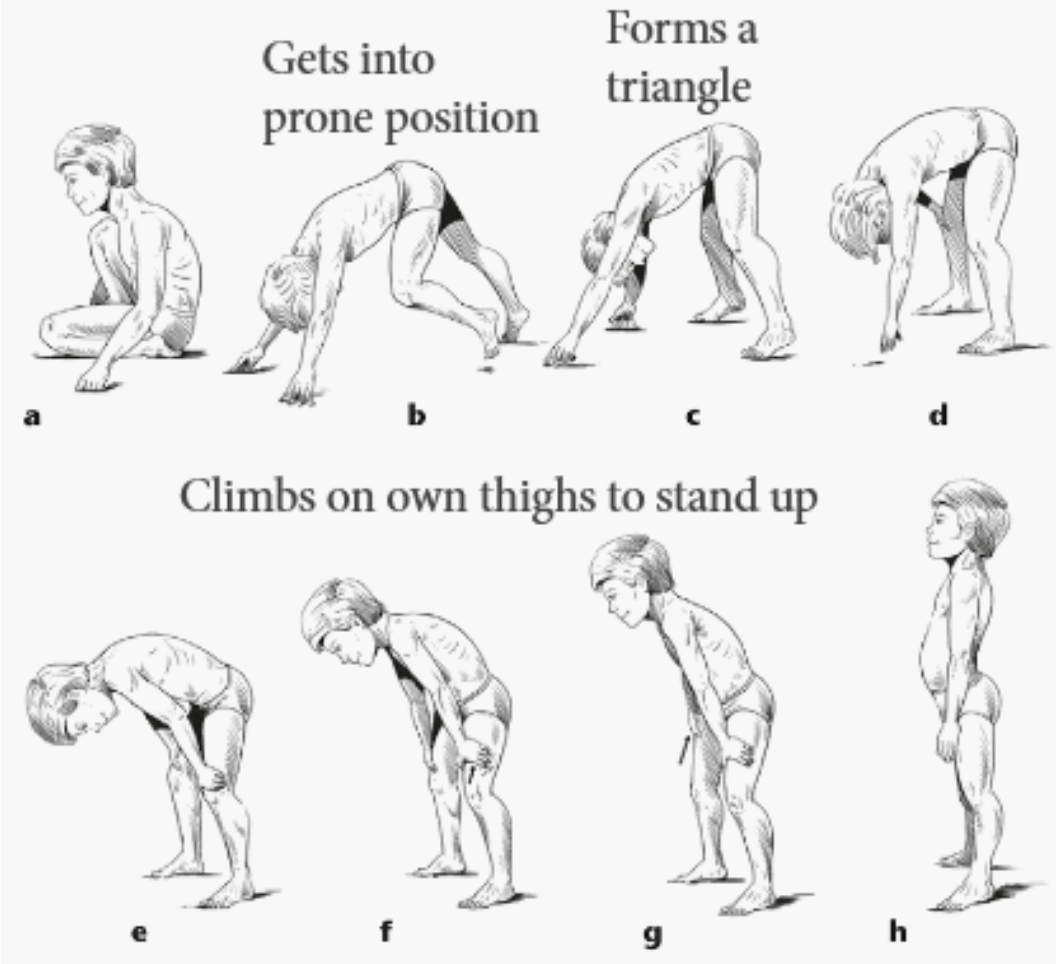

Figure 1: Gower's manoeuvre (13)

MLPA (multiplex ligation-dependent probe amplification) is the most widely used technique. If a pathological mutation is found, the muscle biopsy can be avoided. MLPA, a quantitative technique, can detect exactly which exons are involved, by a copy number variation (CNV) such as a deletion or duplication. This approach exploits PCR working with probes which can hybridise to each of the DMD exons and then amplifying ligated probes. Thanks to this technique, it can be revealed whether or not an exon is present at the genomic level, and in how many copies, not only in male patients but also in female carriers (9). In the case of some small mutations, due to increased risk of prevented hybridization, a second technique is applied to search confirmation (14); this is especially relevant for the confirmation of single-exon deletions. Alternative quantitative techniques include the

oligonucleotide-based array comparative genomic hybridization (array-CGH) allowing to get a full map of CNVs. This method can locate complex rearrangements and mutation breakpoints. A qualitative analysis is represented by next generation sequencing (NGS) that produces millions of copies of the DNA fragments simultaneously. This technique uses a clonal amplification “in vitro” in order to amplify the DNA strand, and sequence its bases with reversible dye-termination. (15)

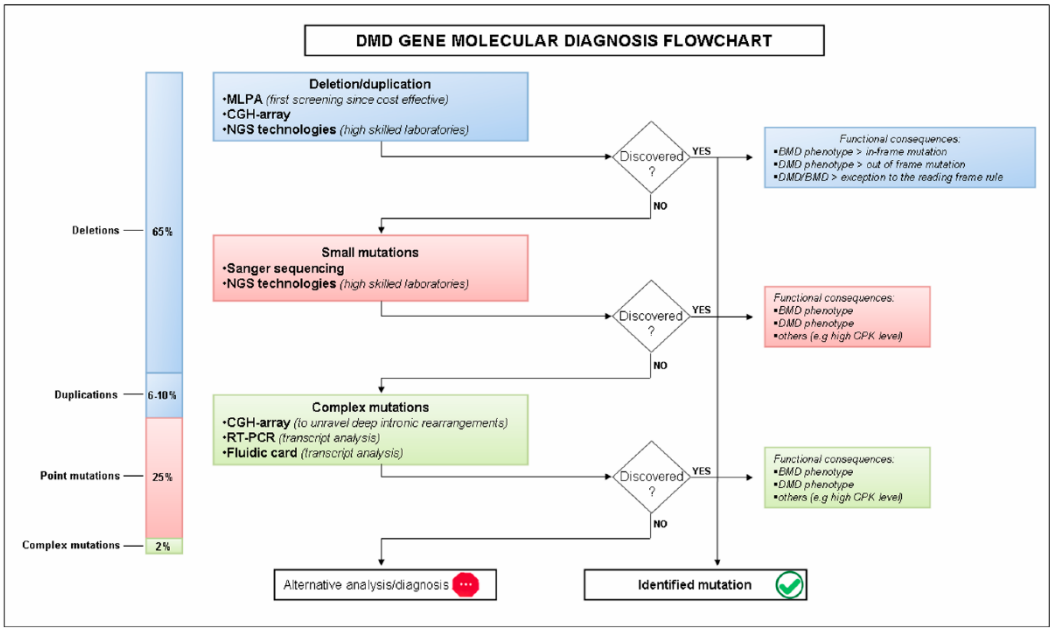

Figure 2: flowchart of DMD diagnosis (15)

At the end of these procedures, it is recommended to provide a genetic consult for the mother and the family, in order to assess the risk of recurrence of the mutation and disease.

Histology

Although muscle biopsy is an invasive technique, it retains a relevant role in the diagnostic process of DMD. In the 1960s and 1970s histochemical stains were introduced in muscle biopsy examination to define myofiber types. Between the 1980s and 1990s, the introduction of immunocytochemistry improved the diagnostic technique for neuromuscular disease by recognising specific sarcolemma proteins and the type of inflammatory cells. (16) However, the muscle biopsy is mandated not only for diagnosis but also for clinical trials for new drugs,

such as exon skipping or gene therapies, in order to examine changes in the muscle and especially in dystrophin expression. (17)

Already in early stages, in haematoxylin and eosin-labelled cross sections on a skeletal muscle biopsy, small clusters of muscle necrosis, degeneration and regeneration with connective tissue (fibrosis) are observed. Consecutively, in late stages, in addition to fibrosis, deposition of adipose tissue is added. (18) Histological features of "dystrophic" muscle include necrotic muscle fibres and regeneration, an early invasion by macrophages, centrally nucleated myofibers, an increased endomysial connective tissue, fat replacement of muscle. (19)

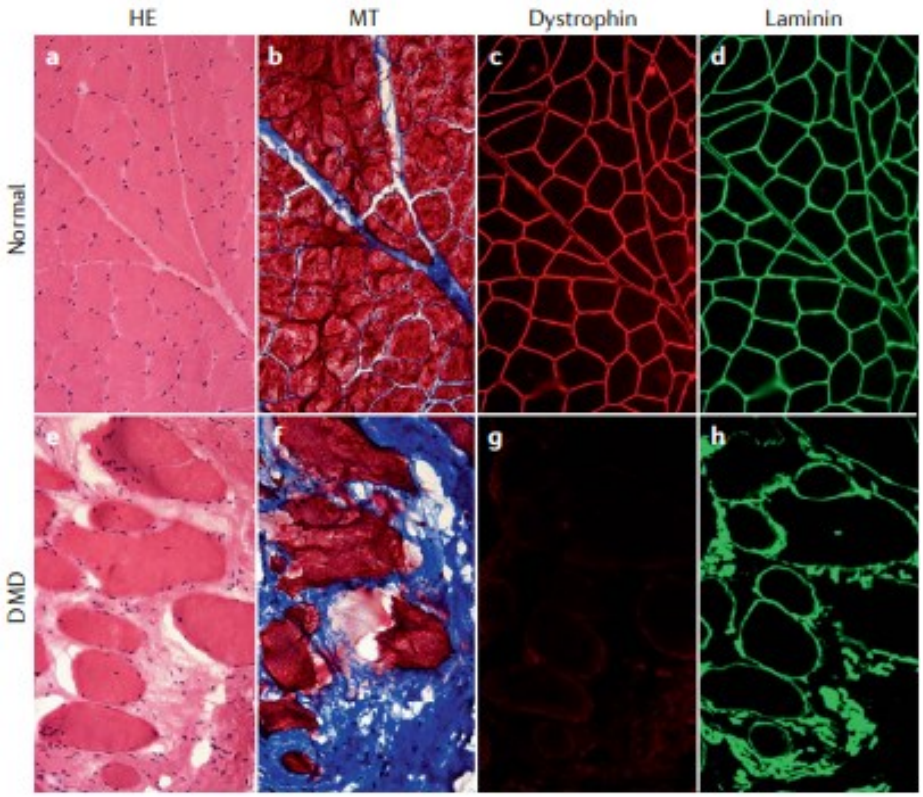

Figure 3: comparison of a section of healthy muscle and skeletal muscle from a patient with Duchenne muscular dystrophy (DMD). Haematoxylin and eosin (HE) staining show the different clinical features described above (panels a and e). Masson trichrome (MT) staining colors fibrotic tissue blue, showing an increased fibrosis in patient with DMD compared with healthy muscle (panels b and f). Immunofluorescence labelling of dystrophin and laminin reveals a lack of dystrophin in a patient with DMD compared with healthy counterpart (panels c and g) and variation in myofiber size (panels d and h). (7)

Clinical History

Duchenne muscular dystrophy is a progressive disorder. The age of onset is usually about two to three years: earlier cases are diagnosed from incidental findings of an elevated value of CK. In patients' early childhood, parents may notice motor delays (as difficulties running), gait alteration, a tendence to fall frequently, and psychomotor retardation. However, with less frequency, difficulties in learning, speech impairment, and behavioural disorders have been reported as presenting signs.

Earlier symptoms include muscle hypertrophy, especially at calves, and proximal weakness which is more prominent in axial and lower limb muscles, causing a typical waddling gate. (12)

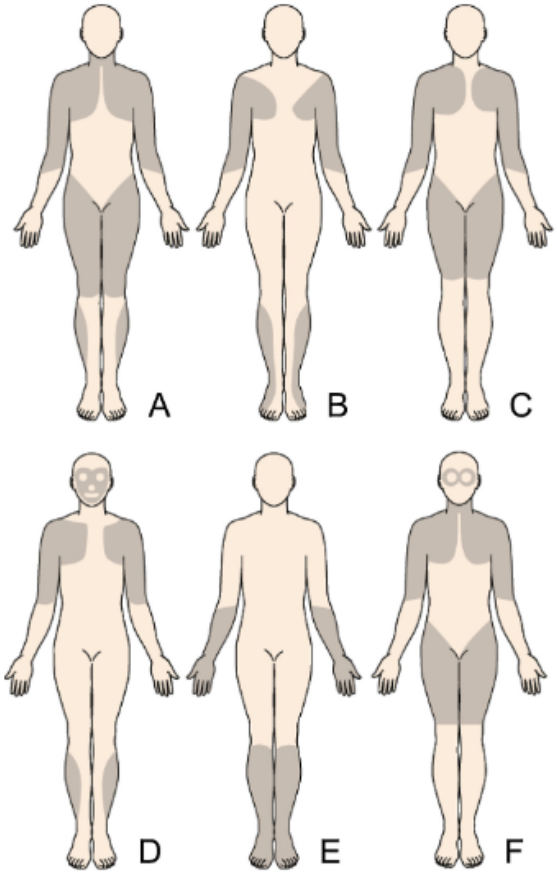

Fig. 4: in this picture is shown the different distribution of the main muscle involvement in different neuromuscular disease. (A) Duchenne, (B) Emery-Dreifuss, (C) limb-girdle, (D) facioscapulohumeral, (E) distal, (F) oculopharyngeal. Affected areas are highlighted in gray. (20)

While walking, patients tend to have compensatory circumductory movements of the arms, in order to keep balance. Some patients may be able to run, but find it increasingly difficult over the years. School-age children are slower in running then their peers.

As short brain isoforms of Dystrophin (Dp140 and Dp71) are expressed in the brain, many patients affected with Duchenne muscular dystrophy have cognitive, neuropsychiatric, and neurodevelopmental disorders, including autistic spectrum disorders (19%), attention-deficit-hyperactivity disorder (ADHD 31%) and obsessive-compulsive disorder (5%). (21) Cognitive impairments affect about one-third of patients. (7) Coexistence of these conditions may suggest the existence of "DMD neuropsychiatric syndrome". (21) Neuroimaging studies have shown that in patients grey matter and total brain volume are smaller, while white matter presents no difference in volume, but an altered microstructural integrity, caused by a disrupted maturation rather than atrophy. (22) In addition to the aforementioned conditions, behavioural disorders are also observed. The cognitive issues observed in dystrophinopathy are in themselves a cause of impaired quality of life and reduced social participation, independently from motor function limitations. Actually, patients have an increased risk of depression and anxiety, especially in the later stages of disease, and need psychological care as well as pharmacological treatments targeted to their specific condition. (23)

As weakness progresses, the loss of muscle strength and moto function lead to major clinical complications. Loss of independent ambulation (LoA) makes the patients wheelchair dependent, usually around the age of 12 to 14 years (in glucocorticoid-treated patients). (24) Imminent LoA is predicted by the inability to walk at least 10m without aid. Wheelchair dependence has relevant consequences on thoracic expansion, and early LoA predicts the chance of developing severe scoliosis, needing a surgical correction.

In the non-ambulatory phase of DMD, cardiac dysfunction and respiratory complications come at the forefront of the clinical picture. The myocardium is affected by dystrophin deficiency, and, similar to skeletal muscle, develops a progressive replacement of contractile cells with fibro-fatty tissue, resulting in ECG

abnormalities, followed by a reduction in contractile force, and usually a dilative remodelling, i.e. dilative, hypokinetic cardiomyopathy. (25) While patients are relatively protected from cardiac symptoms due to their inability to exercise, and despite the availability of cardiological treatments for heart failure, severely depressed systolic function is becoming the leading cause of death in DMD, especially since the widespread use of mechanical ventilation. (26) Weakness of the diaphragm and other respiratory muscles results in restrictive respiratoy insufficiency in DMD, which usually becomes prominent after LoA. Its complications include initially disordered nocturnal breathing, eventually requiring nocturnal ventilation, and subsequently inefficient cough, impaired airway clearance, and increased risk of infections. (27,28)(29).

Management and therapies

The standard of care in DMD currently includes the administration of glucocorticoid corticosteroids. The major benefit of corticosteroids is to preserve ambulation and to slow down muscle degeneration with a lesser degree of fibrosis, especially if started early, around the age of 3 to 5 years. (30) Children are followed with therapy dosage adjustment in order to find a balance between benefit and side effects. The most common side effects in the DMD population consist in increased appetite and consecutive weight gain, exacerbation of osteoporosis and increased risk of fractures, growth stunting, and cataracts.

Since the 1990s, several studies have been done to evaluate the best corticosteroid regimen, among several available (prednisone or deflazacort, and daily vs. several intermittent regimens). An observational study of corticosteroid regimens in the Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS), published in 2015, included 340 participants, comparing the effects on LoA and side effects of Deflazacort (DFZ) and Prednisone (PRED). With both drugs a clear delay of LoA was evident. However, Deflazacort seemed to be associated with later LoA but possibly due to higher dose or an higher adherence to the therapy. (31) Previous studies had suggested a difference in tolerability: prednisone seems to impact weight gain and loss of bone mass more than Deflazacort. (32) Recently, the results of FOR-DMD

(Finding the Optimum Regimen – For Duchenne Muscular Dystrophy), a 3-year randomized controlled trial comparing daily prednisone, daily deflazacort, and intermittent prednisone, were published, definitively confirming the superior efficacy of daily regimens, with similar results for prednisone and deflazacort; the latter was confirmed to induce less weight gain (33) (34)

Other than glucocorticoids, the only other approved pharmacological treatment in Italy and Europe is ataluren, a small molecule aimed at promoting ribosomal read-through of nonsense mutations, which cause about 10-15% of cases of DMD. Despite some controversies during its clinical development, ataluren has shown a clear effect in delaying the progression of muscle weakness in DMD, with good tolerability, and has been approved by the EMA for nonsense mutation DMD from the age of 2 years, limited to the ambulatory phase of the disease (35) (36)

The landscape of experimental treatments currently being tested for DMD is very wide, the most promising strategies being antisense oligonucleotide – induced exon skipping and gene therapy. The field of gene therapy has received great impulse with the development of adeno-associated viral (AAV) vectors, which are capable of effectively delivering genetic material to several tissues, including skeletal muscle and the heart, (37) without integrating into the host genome. (38) Difficulties in DMD gene therapy include the large size of the target organ, therefore needing high vector doses; immunological responses and pre-existing immunity; the size of *DMD* gene, requiring the engineering of a reduced version of the gene (micro-dystrophin), only including functionally indispensable domains. (37) (38) While the first human trials have shown very promising levels of micro-dystrophin expression, safety concerns have arisen concerning several severe adverse reaction, including deaths, due to the activation of innate immunity and subsequent organ (hepatic and/or renal) damage

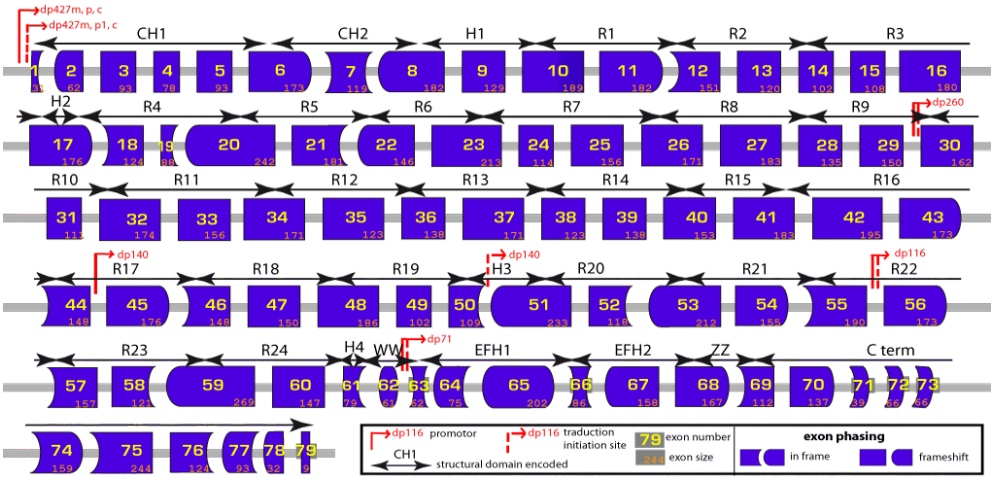

Figure 5: exon representation of DMD. (39)

Due to the genomic structure of the *DMD* gene, schematized in Figure 5, it is often possible, in the case of an out-of-frame deletion, to restore the reading frame of the transcript by excluding (or "skipping") one exon adjacent to the deletion boundaries. The result would be an internally deleted DMD protein resembling Becker muscular dystrophy (BMD) (36) Given the milder course of BMD, clinically it would be convenient trying to convert a DMD phenotype into a BMD phenotype. Despite the promising results obtained in animal models and in Phase 2 human trials, clinical trials has shown limitations and difficulties in establishing clinical efficacy. (40) Details about the main exon skipping AONs are presented below.

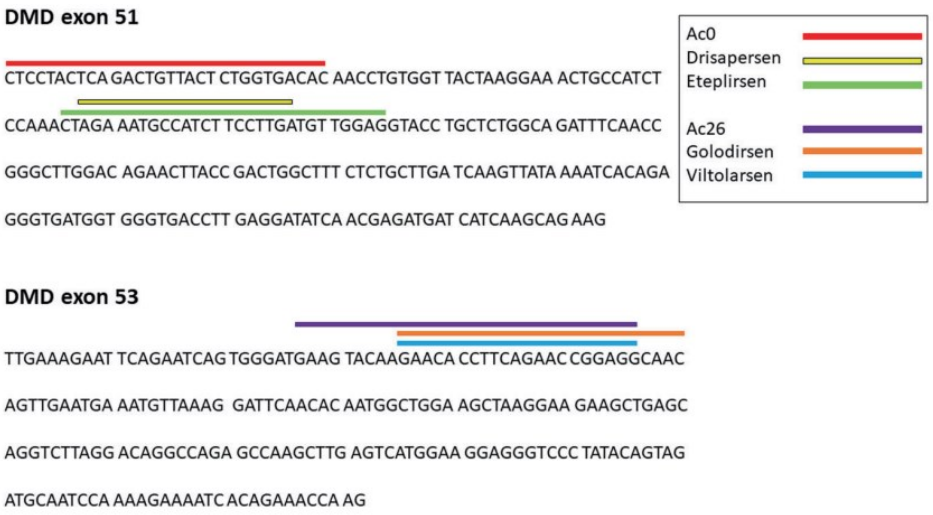

Figure 6: a comparison between the exon skipping therapies. The diagram shows the AON sequence and the complement position of the target region. Ac0 (red) is a 30-mer long morpholino with an optimized sequence complementary for exon

51. Drisapersen (yellow) is a 2OMePS. Eteplirsen (green) is a PMO which binds exon 51. Both Golodirsen and Viltolarsen are PMO which binds exon 51, with different mer length. Finally, also Ac26 is a PMO which binds exon 53. (40)

Eteplirsen

Eteplirsen was approved from the US Food and Drug administration (FDA) in September 2016, on the grounds of evidence of an increase of dystrophin levels in histology; however, FDA required more clinical trials to demonstrate the clinical benefit. (41) It is a 30 nucleotide PMO which hybridizes to exon 51 of DMD, causing its exclusion from the mature mRNA. This process is able to restore the reading frame in several *DMD* deletions, producing shortened functional dystrophin proteins. Eteplirsen might be used in about 20.5% patients, which shows *DMD* deletions ending at exon 50 and starting at exon 52, binding and skipping exon 51. (42) PROMOVI trial (an open-label trial), conducted at 40 sites in the US between November 2017 and June 2019, has enrolled ambulant patients aged 7-16 years that have received a weekly intravenous (IV) infusion of 30mg/kg Eteplirsen for 36 weeks. Though both use of 6MWT and North Star Ambulatory Assessment (NSAA) scale, PROMOVI confirmed that Eteplirsen delayed disease progression, with a good safety profile. (43)

Golodirsen

Golodirsen is an antisense oligonucleotide of the PMO, approved in USA in December 2019. It is designed to induce exon 53 skipping, thus enabling the treatment of about 8% of all DMD patients. Like Eteplirsen, its mechanism of action is based on an increase in dystrophin production in skeletal muscle. (44) In a multicenter trial, which assessed Golodirsen safety for up to 189 weeks and efficacy over 144 weeks, has demonstrated that Golodirsen at dose of 30mg/kg/week is well tolerated and promotes an increase of about 1% of dystrophin protein,. However, clinical trials are complicated by the relatively small numbers of patients with eligible genetic mutations. (45)

Viltolarsen

Viltolarsen is a phosphorodiamidate morpholino antisense oligonucleotide designed to bind to promote skipping of exon 53 to skip it and restoring the open reading frame. It received the first approval in March 2020 in Japan. (46) It works for 8% of DMD patients, who carry compatible mutations. Clinical trials are still running to demonstrate efficacy of Viltolarsen. For instance, the RACER53 trial plans to study efficacy of Viltolarsen on motor function tests. The trial aims at recruiting 74 participants to receive Viltolarsen during 48 weeks. (47)

Phenotype variability in DMD

The phenotype of Duchenne muscular dystrophy is not completely homogeneous among patients, with a relatively large inter-patient variability, which make it difficult to formulate prognostic predictions, and quantify responses to therapies in both a research and clinical setting. While some of the variability in DMD phenotype may be attributed to the environment, e.g. quality of care, socioeconomic status, etc., several genetic causes for variability have been characterized, which may be divided in to “cis” and “trans” genetic effects.

“Cis” effect – DMD mutations

The most common modifications of the DMD gene (65% of cases), followed by duplications (5-15%). Two deletion hotspots are more frequent than others: the first is located towards the centre of the sequence and includes exons 45-55; the second is located towards its 5’ end and includes exons 2-19. (8) But not all DMD mutations exert the same effect on phenotype (48,49) (50) For example, it has been demonstrated that exon 51 skipping induce earlier LoA compared to all other mutations (49). From that, it can be assumed that endogenous exon skipping follow the production of low levels of dystrophin, addressing few in-frame mutations.

"Trans" effect – Genetic background

In addition to the effect caused by pathogenetic mutations, the phenotype of DMD may also be modulated by variants in genes different from *DMD*. These may be common variants, not associated with pathology in the general population. Indeed, different single nucleotide polymorphisms (SNPs) in five different genes have been associated with phenotypic variability in DMD. These genes may be called "genetic modifiers": *SPP1*, *LTBP4*, *CD40*, *ACTN3*, and *THBS1*. (51) Identification of these genes could be a major turning point. Primarily it could influence the new target therapies, then help to do a better diagnostic framing and better follow-up.

SPP1 (osteopontin-OPN, Secreted PhosphoProtein 1)

SPP1 is the first identified modifier of DMD, mapped on chromosome 4q21-q25. (52) Single nucleotide polymorphisms (SNPs) in *SPP1* have been associated on phenotypes. (51) *SPP1* codes for ostepontin, a glycoprotein which plays a role in several biological processes, including tissue repair and regeneration in muscle, bone remodelling, and cell-mediated immunity. An overexpression of OPN is implicated in an activation of inflammatory processes. (52) In a stud, of two DMD cohorts of patients (Padova cohort with 106 patients with DMD and Cooperative International Neuromuscular Research Group cohort with 156 patients), *SPP1* has been associated to more severe DMD phenotypes, depending on rs28357094 ($p = 0.001$ in Padova cohort), a SNP promoter with 66bp upstream of the transcriptional site, predicted to alter transcriptional efficiency. Specifically, an association was found between a more severe phenotype and the minor G allele. In both cohorts, the minor G allele was found to be associated with a more severe phenotype, not only in ambulation but also in grip strength. DMD patients carrying the G allele are significantly weaker and lose ambulation about 1 year earlier, in a population treated with corticosteroid therapy. (53) The OPN is apparently involved in complex roles in DMD pathology. At the same time, OPN may exacerbate fibrosis due to an increase in NF-kB activation, but also participates in tissue repair after damage. (51)

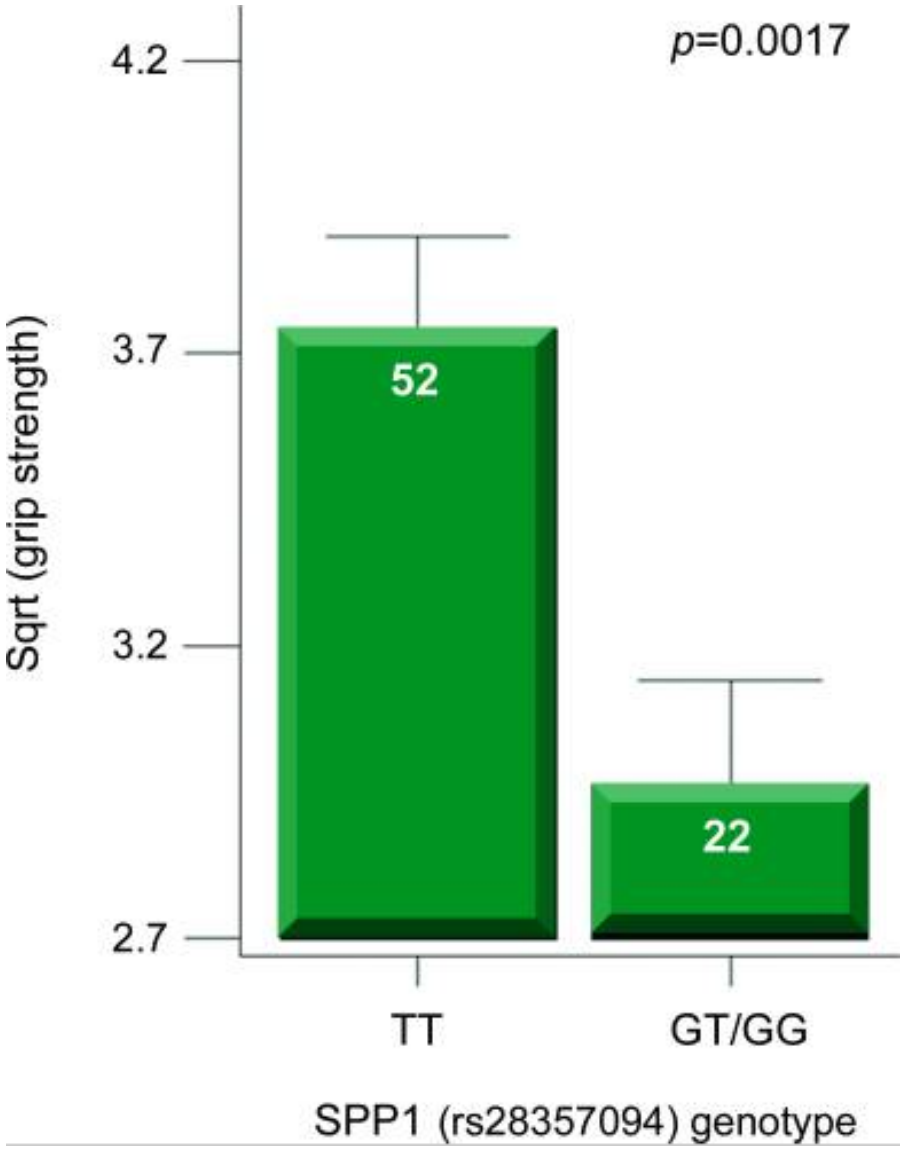

Figure 7: SSP1 genotype is associated with decreased grip-strength in steroid-treated patients with DMD. The graph represents the strong association with SPP1 genotype and strength in steroid-treated CINRG cohort. (53)

After the first studies described above, there were some contrasting findings across different studies. It has been suggested, based on results in the CINRG-DNHS, the the effect of *SPP1* may be more evident in glucocorticoid-treated populations, i.e. that *SPP1* modulate response to this treatment rather than the disease phenotype directly. (54)

LTBP4 (Latent Transforming Growth Factor *β* Binding Protein 4)

LTBP4 is a matrix protein that binds TGFβ in a latent complex in the extracellular matrix, coded by the same *LTBP4* gene. The homologue murine gene *Ltbp4* has been identified by a genome wide mapping in murine muscular dystrophy models as a modifier of disease severity. Belonging in the TGFB binding protein family, LTBP4 forms the "latent" LTBP4- TGFβ complex, which sequester TGFβ and prevents it from reaching its receptors at the surface of cells. In humans, four SNPs along the *LTBP4* gene give rise two major haplotypes encoding the VTTT and IAAM (after one-letter codes for varying aminoacids) protein isoforms. In the UDP severe dystrophinopathy cohort of 254 participants, the homozygous haplotype IAAM was associated with LoA delay of approximately 1.5 to 2 years (51). In contrast to VTTT, IAAM isoform is associated with increased muscle preservation, reducing dystrophin muscle damage. (55) The underlying mechanism is probably that the minor haplotype IAAM leads to a more stable latent complex, and therefore reduced TGFβ signalling and an attenuation of fibrosis (56) The effect of LTBP4 was validated in several independent cohorts, and should be considered for DMD diagnosis, prognosis and therapies. (57)

CD40 (Tumor Necrosis Factor Receptor SuperFamily Member 5)

CD40 is expressed on undifferentiated of B cells, playing an essential role in costimulatory signal for proliferation, development, and activation, mostly for promotion of humoral immune response. Before entering in lymphoid germinal centres, CD40 expressed in B cell requires interaction with its ligand CD154. Once activated, B cells secret high-affinity antibodies. (58) The blockage of interaction between CD40 and CD154 has improved many study prospects in autoimmune disease as Myasthenia Gravis. (59) CD40 was established as a DMD modifier by a cohort comprehensive of patients from the CINRG-DNHS, Padova University, United Dystrophinopathy Project, and the Bio-NMD consortium. An association with earlier LoA in DMD was established with the rs1883832 minor T allele, which downregulates CD40 signalling, consequently causing a failure of regeneration of skeletal muscle. The effect of this SNP on age at LoA has been estimated to be around 1 year. (60) (56)

ACTN3 (Actinin-3)

ACTN3 codes for actinin-3. Alpha-actinin-2 is the principal component of the Z-line in the sarcomere in the skeletal muscle, binding the actin filaments. Whereas actinin-3 is an isoform of actinin, expressed in fast fibres (fibre IIb). Alpha-actinin-3 deficiency, due to a common nonsense polymorphism (R577X, rs1815739), causes on the one hand a decrease in sprint and in muscle mass, on the other an increased endurance performance in athletes. (61) In the study by Yang and colleagues conducted on athletes, it emerges that the presence of alpha-actinin-3 in fundamental for sprint performance. Athletes with a higher frequency of the 577R have an increase sprint performance. On the other hand, athletes with a 577XX genotype, i.e. a complete lack of alpha-actin-3, show enhanced endurance performance. (62) Furthermore, rs1815739 has been investigated in a study with a cohort of DMD patients (n=272) enrolled in the CINRG-DNHS. In knockout (KO) mice for ACTN3 there is a shift from the properties of fast fibre to slower fibre, such as a reduced fibre diameter and increased oxidative metabolism due to an activation of calcineurin. This shift could be protective for DMD muscle, especially because of calcineurin overexpression. Also in this study, it is shown that 577XX (null polymorphism) ACTN3 leads to both reduced muscle strength in both mice and humans, and protection from eccentric damage. The effects on LoA are controversial (earlier LoA in 577RX heterozygotes).

Histopathology allows to understand better investigate the underlying mechanisms. As in figure below, the main differences between the double knockout model (dKO, both dystrophin- and ACTN3-deficient) and the dystrophic mice model (mdx) are the consistent reduction in necrosis and an increase in centrally nucleated fibres in the first one considered.

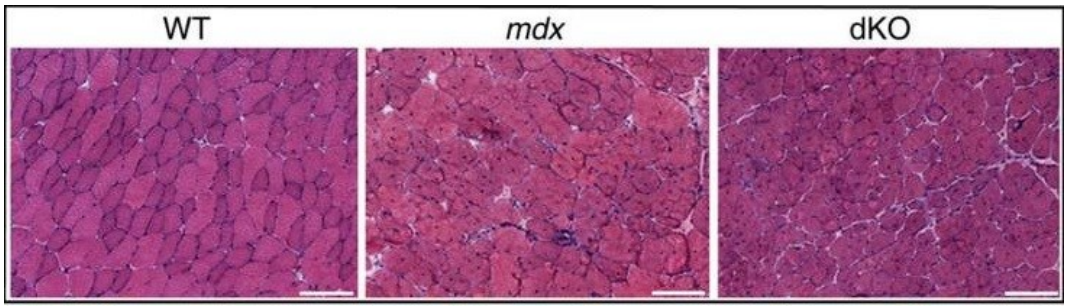

Figure 8: TA section in haematoxylin-eosin which compares WT, mdx and dKO muscle.

Nevertheless, role of alpha-actine-3 in DMD patients remains controversial. (61)

THBS1 (Thrombospondin-1)

Two significant SNPs (rs2725797 and rs2624259) within a LD block emerged from a genome wide association study (GWAS) conducted in the UDP severe dystrophinopathy cohort. This SNP was shown to be a long-range regulator of thrombospondin-1 expression. (55) Like other gene modifiers previously mentioned (SPP1 and LTBP4), it is involved in the TGF-B activator pathway and interacts with ones of the gene modifiers itself, LTBP4. (63) The minor allele of rs2725797 is found to be strongly associated with LoA variability in DMD patients (recessive model p-value = 6.6×10−9; additive model p-value = 7.5×10−6), delaying LoA. Especially, rs2725797 has been associated with reduced THBS1 expression in the muscle tissue. (55)

Aim of the study

As explained in the Introduction, the relevant phenotypic variability observed in DMD has only partially been explained by the known genetic modifiers. A precise characterization of DMD genetic modifiers would allow better prognostic predictions and patient/family counselling; better clinical trial design, stratification, and interpretation; and possibly point to novel therapeutic targets. Genetic modifiers identified so far have mostly been described in candidate-gene studies; i.e. there was previous knowledge about a role of those genes in dystrophic muscle pathology. Conversely, the unbiased approach of a Genome Wide Association Study (GWAS) would allow the identification of novel loci, with potentially unsuspected mechanistic properties. However, GWAS requires large patient populations in order to reach adequate statistical power, which are difficult to collect in rare diseases such as DMD

Therefore, we aimed to collect a large multi-centric cohort of DMD patients from the Italian DMD Network, perform genome-wide genotyping, and associate genetic variants with age at LoA.

In this thesis, preliminary results of a simple size of 490 patients are shown, analysed by using a classic Genome Wide Association Study (GWAS) approach for the examination of common variants. A larger investigation, which requires a cohort of 700+ individuals, will cover the examination of rare variants by applying Sequencing Kernel Association Test (SKAT) methods (not included in this thesis). (64)

Material and methods

Recruitment of eligible DMD individuals at each Institution

Thanks to the Italian DMD Network (i.e University of Padova, University of Milan, "Mondino" Institute in Pavia, IRCSS "Medea" in Bosisio Parini, "Besta" Neurological Institute in Milan, University of Turin, "Gaslini" Institute in Genova, IRCSS "Bellaria" in Bologna, University of Pisa, "Bambin Gesù" Hospital in Rome, Catholic University of the Sacred Heart in Rome, University "Vanvitelli" in Naples, Neuromuscular Omnicenter NEMO in Messina and University of Messina) clinical data and DNA of a large cohort of DMD patients, evaluated with validated and standardized methods, have been collected. Importantly, this same network has largely contributed to the development and validation of clinical scales currently used in DMD follow-up and clinical trials, and to the current knowledge on DMD natural history and progression.

Thanks to the collaboration with each centre, we have been able to collect clinical data of 734 patients. After getting their informed consent, patients could donate their blood sample, from which we extracted and store DNA samples.

The following were the inclusion/exclusion criteria:

1- Availability of a DNA sample and signing of informed consent on participation in the research.
2- Males with Duchenne Muscular Dystrophy with a null DMD mutation (i.e duplications, frameshift deletions and small rearrangements)
3- No dystrophin expression or a very low amount levels (i.e., 0-5%) examining by the immunohistochemistry (IHC) or western blotting; however the presence of a muscle biopsy was not compulsory, in the presence of a DMD-compatible phenotype and an out-of-frame mutation.
4- For each DMD patient, availability of clinical information useful for the purpose of the association study: ambulatory status, age at LoA (for non-ambulatory patients in the absence of this, age of the last visit) and

information about glucocorticoid treatment (at least 1 years before LoA vs. less or no treatment, as a dichotomoic variable).

5- Patients enrolled should not include siblings or cousins of subjects already involved in the study. The involvement of related individuals contributes to an important population stratification bias to be avoided on GWAS, because they share similar mutations and environment.

Sample Genotyping

Each sample is expected to provide 900 ng of genomic DNA. Genotyping several SNPs allows genome-wide coverage. The ideal platform for the simultaneous determination of the genotype of multiple SNPs is a microarray. The Illumina Infinium Omni2.5Exome-8 version 1.5 chip was employed, in collaboration with Biodiversa s.r.l, Rovereto (TN)..

Infinium assay for genome-wide genotyping

The surface of every chip retains from hundreds of thousands up to a million of beads, each inserted in an appropriate well. Every bead is complementary to a specific genome locus, through a certain oligonucleotide probe that coats bead. Therefore, the genome, amplificated and fragmented, hybridizes with the corresponding oligonucleotide probes on the chip. Each of the four different nucleotides is labelled and once paired with the site of interest, probes emit signals of different intensities, captured, and recognized by an appropriate scanner. Thanks to the fluorescence intensity of each fluorophore, the allelic ratio of each SNP, and genotype, can be defined. Each patient is reported in a file composed of three columns: in the first column are expressed the SNPs in alphabetical order, next in the second and the third are shown individuals specific codes and the observed values of the two alleles for that SNP.

GWAS

After genotype calling is essential to continue with data cleaning due to avoid bias in GWAS, caused by wrong genotypes, wrong phenotype data, and wrong association. (56) GWAS is based on the significant statistical association between a given phenotype and a genotype of our interest: something in genetics is having an impact on phenotype. GWAS is an approach that involves in scanning bio markers such as SNPs from people sample of DNA in order to find genetic modifies, associated with a specific phenotype.

Data cleaning and quality control steps include: an application of a missing-call threshold of 0.05 (both by SNPs and by individuals); a threshold of 0.1 for duplicates and siblings; Hardy-Weinberg Equilibrium deviations. Moreover, to check GWAS results a Quantile-Quantile (QQ) plot is used for systematic bias.

With the complete investigation we aim to analyse main phenotypes of DMD, collected during periodic visits, with GWAS method: loss of ambulation, North Star Ambulatory Assessment (NSAA), 6 minutes walking test (6MWT), Timed Function Test (TFTs), upper limb function, respiratory insufficiency, and dilated cardiomyopathy. However, being a preliminary study, this thesis is based only on the evaluation of LoA and its association with common variants, with a MAF>10%. Rare variants cannot be analysed with GWAS, but we will apply SKAT methods, which perform better with a lower MAF.

The model applied to analyse LoA is a Cox Regression model, which is a statistical method able to associate several variants with time-to-event, in this case with age at LoA. In particular, the time variable was represented by age, and event was LoA, with all patients who were still ambulatory regarded as “censored” at the age at last evaluation. This model is useful in medicine and preferred over linear regression, especially when considering time-sensitive measures, while adjusting for several variants playing as confounding effect.

Covariates included in the model were the following: glucocorticoid (GC) treatment (at least 1 year before LoA, vs. less or no treatment); in-frame vs. out-of-frame mutations, as some patients who are defined as DMD may still have in-

frame mutations, representing exceptions to the reading frame rule; and the first two principal components (PC1 and PC2) from a principal component analysis of genotypes. The inclusion of these covariates should lower the risk of incurring into bias. The inclusion of PCs as covariate allows to partially adjust from population stratification deriving from the inclusion of a few patients of non-Italian ancestry.

Bioinformatic workflows used includes Centos 8, with 8 vCPUs (Xeon E5-2680v4), and 32 GB of RAM. Therefore, implementing by the application of PlinkCoxSurv, a function in R, it is allowed us to test for SNP-covariate association. Also the qqman, tidyverse, ggpubr, MASS, and gwasurvivr in R version 4.0.4 were applied. Thus, the algorithm generates the model:

$$\sim a + b + c + SNP + c \times SNP$$

Where a and b are covariates, and c is the interaction term.

Loss of Ambulation (LoA). LoA was defined as the age at which the patient lose completely the ability to ambulate, that is, when continuous wheelchair (manual or electrical) use is necessarily.

Survival Analysis. Considering LoA as the event and age in years as the time variable, we managed to make a time-to-event analysis by using the "survival" package on R version 4.0.2. We also distinguished patients by the most significant SNP resulted from GWAS, that is rs10797961.

Kaplan – Meier analyses

The Kaplan – Meier estimator is a statistic curve used to approximate the survival function, usually considering the Time (expressed in years, months, or days) and a Percentage of Survival. Our clinical data has been set in a Kaplan – Meier curve, where on the horizontal axis the age at LoA is placed such as Time to Event, and on the vertical axis is placed the Survival Probability. Through this tool, median ages at LoA and corresponding 95% confidence intervals were estimated. The curve will show more or less steep trend depending on modifier factors.

Illumina infunium Omni2.5Exome-8 version 1.5 chip8

To genotype all patients participating in the study, an Illumina Infinium Omni2.5Exome-8 version 1.5 chip8 (with 2,618,000 SNPs) was employed. Indeed, Illumina is one of the most popular and used commercial designs using the 50-mer sequence adjacent to the SNP and binding different alleles. Through the hybridization, different fluorescence intensity signals are produced to discriminated different alleles, corresponding to each SNP of the array. (56) Thereafter, fluorescent signals are read by fluorescence microscopy. An Illumina iScan tool in the Biodiversa s.r.l Center was employed to genotype. Then, the GenomeStudio Software can pair genotypes with each SNP position.

Results

Cohort statistical inference

In this preliminary study, we have available both DNA and clinical data of 490 patients. In these patients, deletions are the most represented (68%) followed by small mutations (23%) and then duplications (9%). Some patients, thanks to their genetic mutations, could be undergoing treatment of exon skipping.

These patients were analyzed with Cox Regression, considering the following covariates: glucocorticoid (GC) treatment, in frame vs. out of frame mutation, and PC1 and PC2 (principal components).

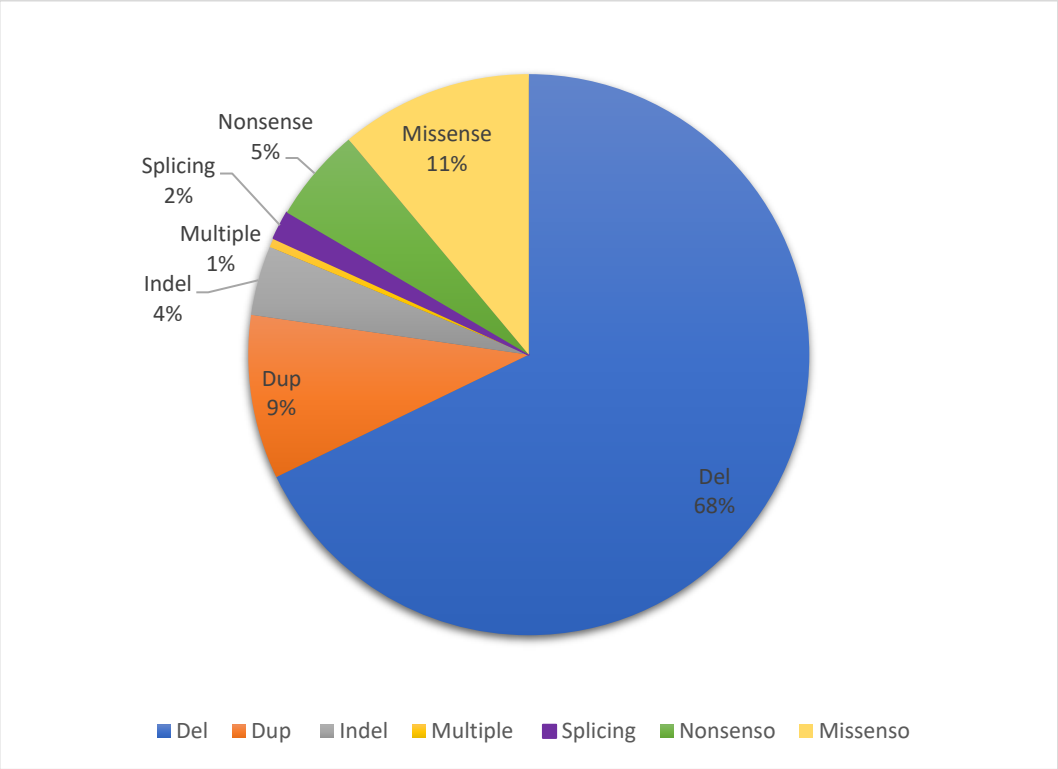

Figure 9: percentage distribution of mutations

Principal component analysis

Principal component analysis (PCA) was applied to identify two principal components (PC1 e PC2), through the function -pca in PLINK. The role of these two PCs is to adjust for ethnic variability, and therefore to correct the population stratification. PCs are linear transformations of the original independent variables, that allow for simplification and streamlining of the evaluation. Data of foreign patients act as confounding factors and contribute to population stratification, then to bias. In the plot below, a cluster with the lower PCs has been shown, and it is represented by Italian patients. Data points to the right correspond to patients of non-Italian ancestry.

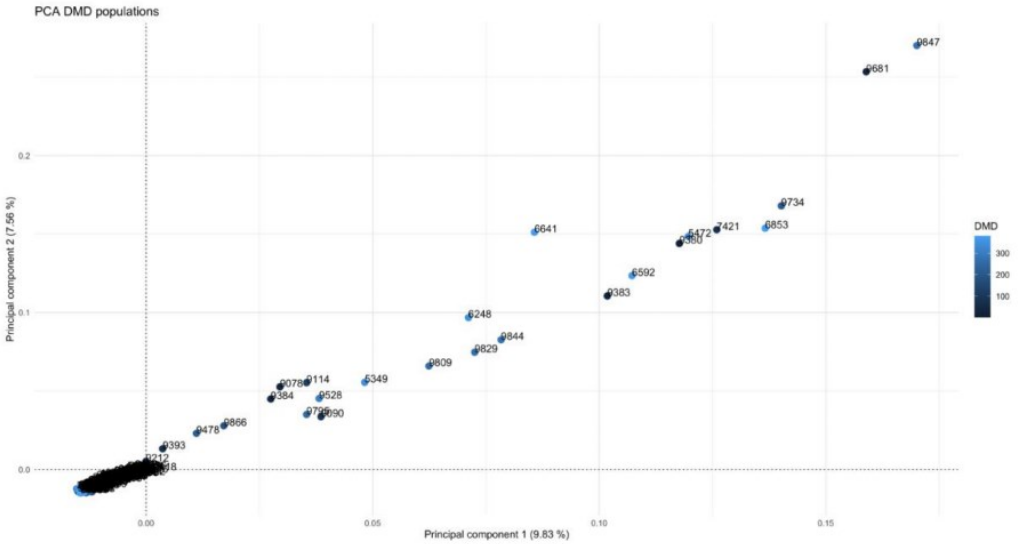

Figure 10: plot showing the distribution of DMD patients depending on principal components, identified by a PCA.

Survival analysis

To analyse this data, we employed a Survival Analysis using a Kaplan-Meyer estimator, where the event is the age of LoA. In the 490 patients presented in this analysis, the median age at LoA was 11.2 years.

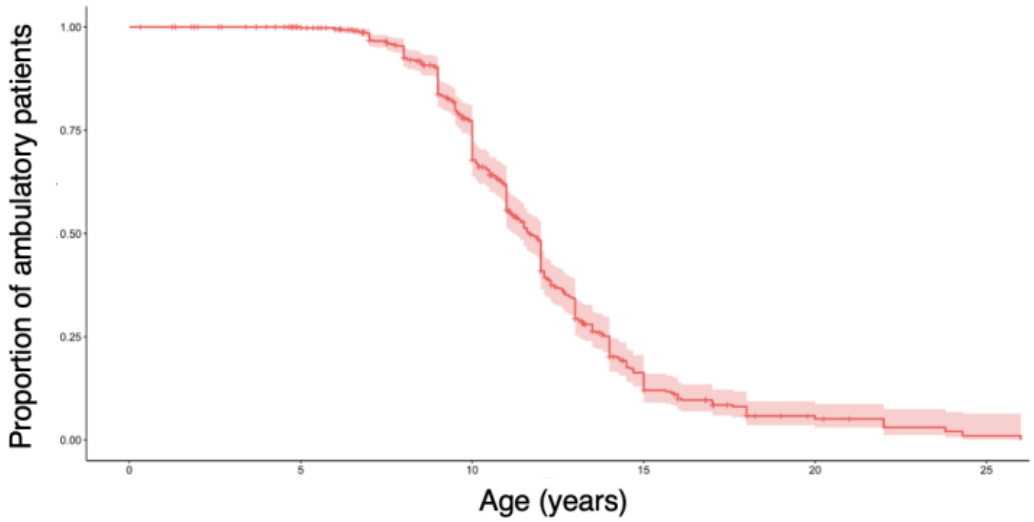

Figure 11: Kaplan-Meier plot of LoA of 490 patients.

GWAS result

W employed the Cox proportional hazards method to analyse SNP effects: GC treatment, in-frame vs. out-of-frame mutation, PC1 and PC2 were used as covariates. A MAF filter was set at >10%. As no SNPs reached the genome-wide significant threshold of $5x10^{-8}$, we set a "suggestive" p-value threshold at $1x10^{-5}$. Observing the Manhattan Plot, 16 SNPs exceed this threshold value. Top association signals are reported in Table 1. The most significant signal among them corresponds to rs10797961.

The significant signals of chromosome 1 reveal a locus of 15 SNPs tagging a region of 16,348bp. This locus spans the entire gene *C1ORF21*, but rs10797961 is located 3,800 bp upstream from it.

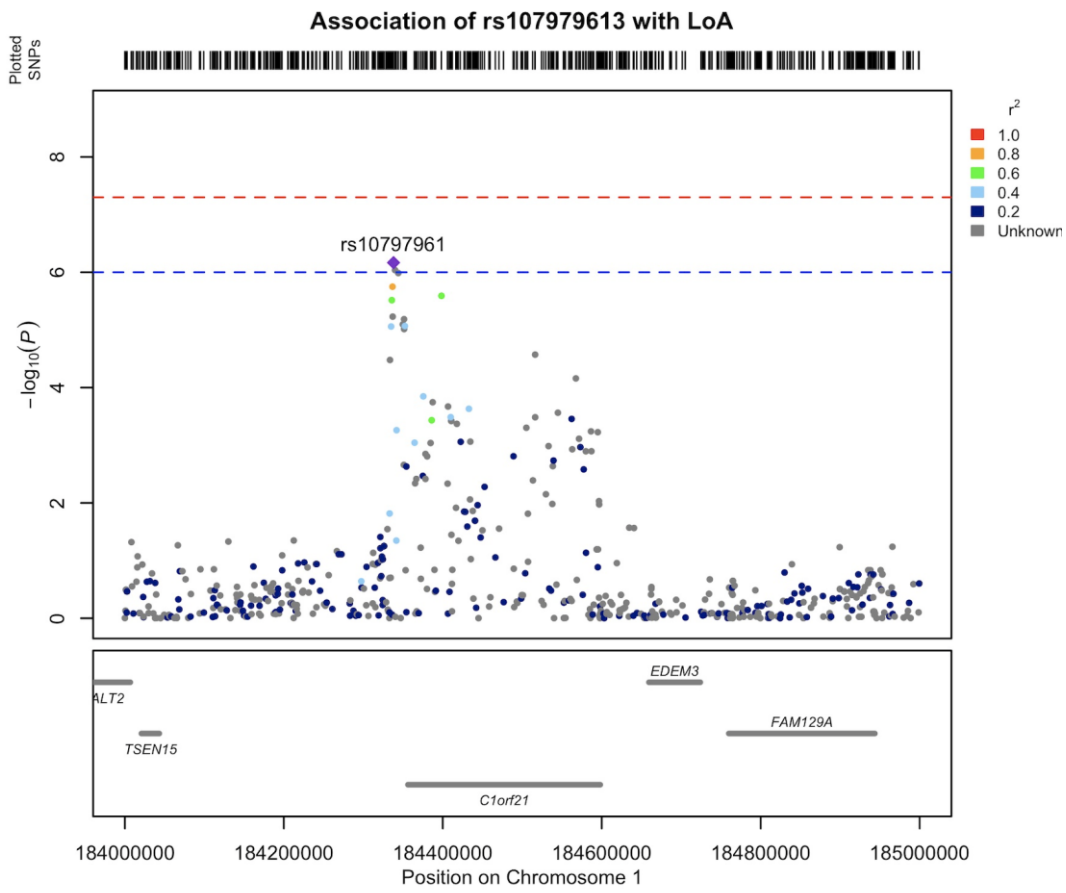

Figure 12: association of rs10797961 with LoA

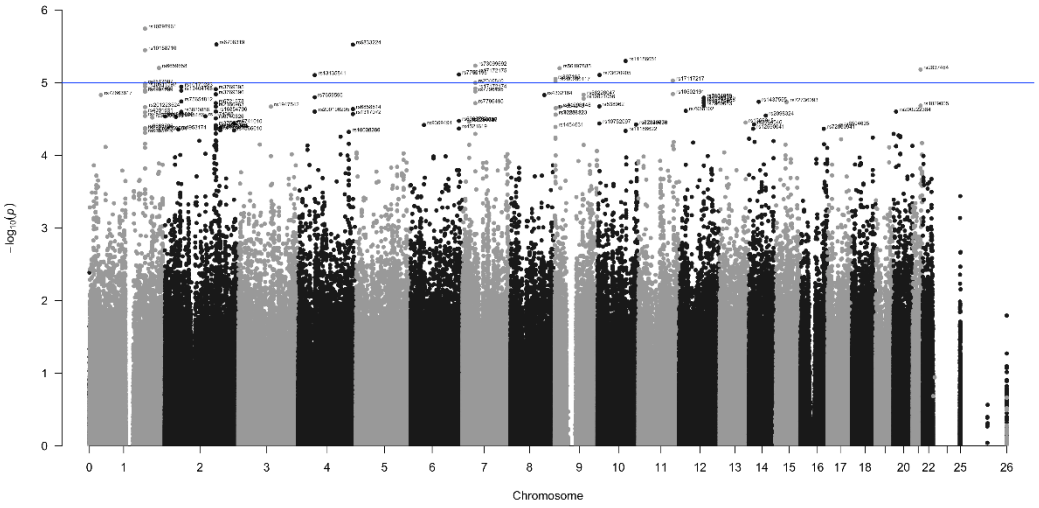

Figure 13: Manhattan Plot of GWAS of LoA (Cox model with 490 patients). Frame, GC treatment and PC1-2 as covariates. MAF>10%. A cluster of 53 SNPs can be highlighted.

SNP	*Allele1*	*Allele2*	*Weight*	*Zscore*	*P.value*	*Direction*	*CHR*	*BP*	*P*
rs10797961	a	g	489	-4.774	1.80e-06	--	1	184338037	1.80e-06
rs6708319	a	t	486	-4.672	3.00e-06	--	2	170958600	3.00e-06
rs6833224	t	c	489	-4.671	3.00e-06	--	4	181316450	3.00e-06
rs10158710	a	c	490	-4.635	3.60e-06	--	1	184398346	3.60e-06
rs11186651	t	g	490	-4.564	5.00e-06	--	10	93424883	5.00e-06
rs73099692	t	c	490	4.532	5.80e-06	++	7	43262410	5.80e-06
rs6659658	a	g	490	4.517	6.30e-06	++	1	230272380	6.30e-06
rs56197885	t	c	489	4.515	6.30e-06	++	9	16176541	6.30e-06
rs2837464	a	g	490	4.506	6.60e-06	++	21	41529188	6.60e-06
rs17172175	a	g	489	4.485	7.30e-06	++	7	43266385	7.30e-06
rs7750196	a	c	490	4.473	7.70e-06	++	6	159864864	7.70e-06
rs73620905	a	g	151	-4.469	7.80e-06	?-	10	7467299	7.80e-06
rs13135841	t	c	490	-4.468	7.90e-06	--	4	55045739	7.90e-06
rs897165	t	c	490	4.443	8.90e-06	++	9	2710537	8.90e-06
rs17117217	t	g	490	-4.431	9.40e-06	--	11	114465917	9.40e-06
rs200280117	t	g	489	-4.427	9.50e-06	--	9	2707850	9.50e-06
rs2040840	a	g	490	-4.415	1.01e-05	--	7	43274439	1.01e-05
rs4512607	t	c	489	-4.409	1.04e-05	--	1	184351279	1.04e-05
rs10911592	a	c	486	-4.391	1.13e-05	--	1	184352288	1.13e-05
rs10170283	a	c	339	-4.389	1.14e-05	-?	2	54956566	1.14e-05

Table 1: top signals from SNPs are annotated. SNP rs10797961 in chromosome 1 reaches the most significant p-value.

The forest plot below (Figure 16) indicates a Hazard Ratio (HR) of 1.87 (95% CI: 1.48 – 2.40), for the heterozygous genotype (AG) or the homozygous genotype (GG), in a dominant model. On the other hand, GC treatment shows a HR of 0.47 (95% CI, 0.37 – 0.6). The meaning of these coefficients is that, by unit of time (e.g. per year), GC treatment reduces the probability of LoA by 53%, while the SNP increases it by 87%.

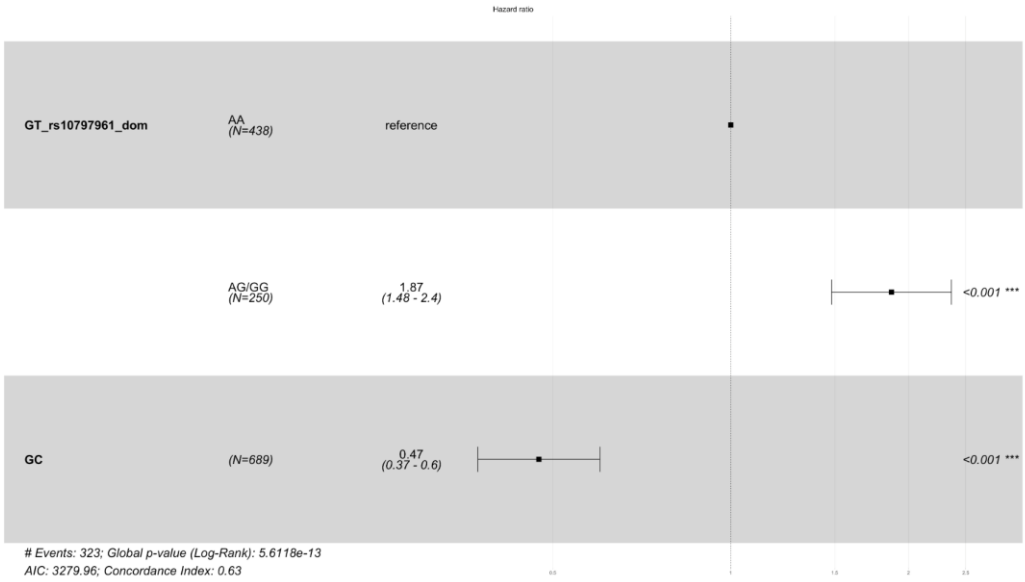

Figure 14: Forest-plot showing effect sizes (Hazard Ratios) on LoA of genotype and GC treatment. Sample size differs from the GWAS analyses, in which some patients had been excluded due to missing covariate data. The effect of SNP has been evaluated on a larger court.

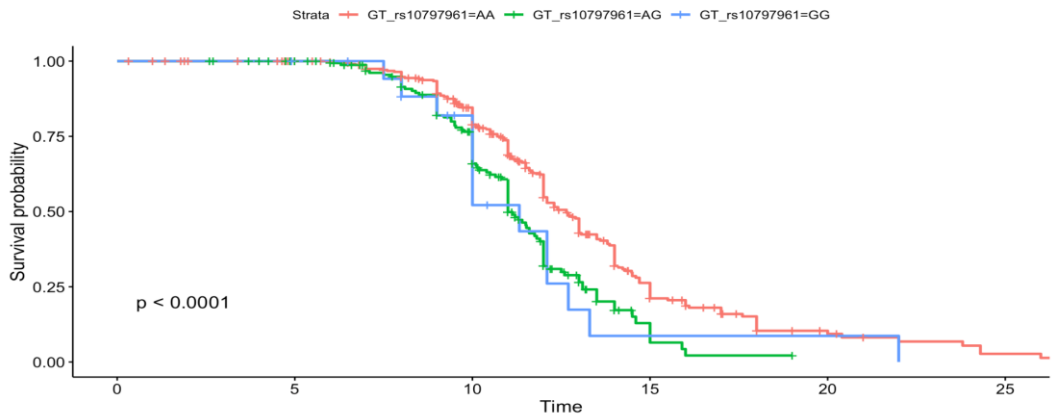

Figure 15: a Kaplan Meier plot of LoA based on an additive model. The curves for AG and GG have an almost overlapping trend, and demonstrate earlier age at LoA compared with AA. The effect of SNP has been evaluated on a larger court.

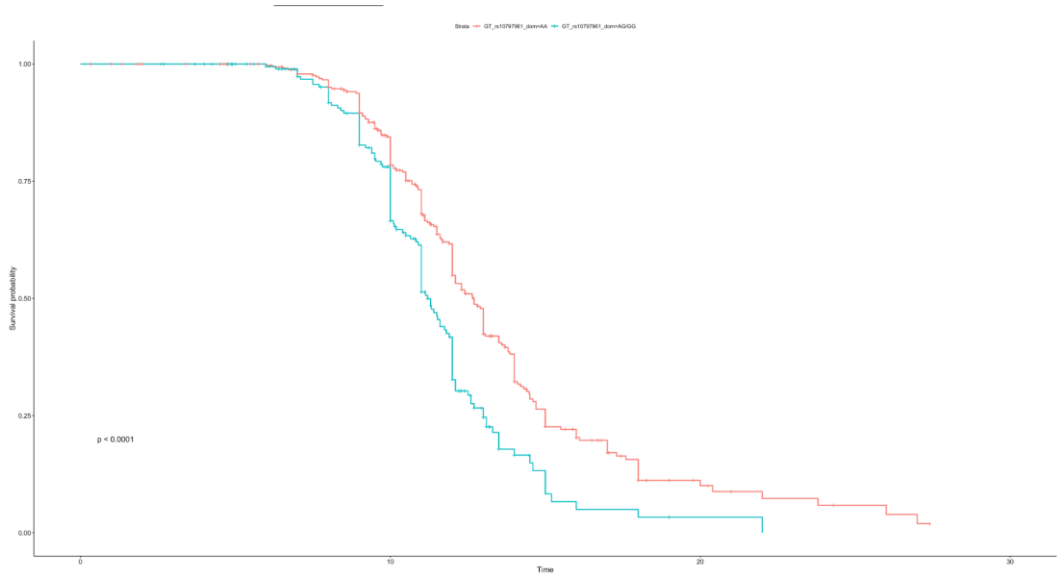

Figure 16: Kaplan Meier (dominant model)

Discussion and conclusion

This thesis stands as a preliminary study of a larger one to be completed soon, which includes a cohort of 700+ patients. We set ourselves the goal of a GWAS, based on a Cox Regression Model, in which each SNP was represented by a quantitative variable, in an additive model: i.e AA-AG-GG as 0-1-2 copies of the minor allele. Age at LoA has been applied as Time To Event, because it is clinically relevant and one of the most significative data following the development and severity of the disease. The rare variants (with a low frequency), and other phenotypes (i.e respiratory/cardiac function measures) will be examined in the definitive study. Meanwhile, data collection, genotyping, and data analyses are now continuing to ensure a more effective analysis with conclusive data. Possibly because of the relatively smaller sample size (490 patients vs. 700+) there were no value that reached the genome-wide significant p-value threshold of $5x10^{-8}$, but despite this, we can already draw attention to a significant locus, *C1orf21* which has been tagged by SNPs both upstream the gene, probably regulating its expression, and across the entire genomic region containing the gene. This gene codes for a protein that has not been entirely characterized, but that does have some functional annotations: it acts as a carrier between cytoplasm and nucleus, and it appears to play a role in cell proliferation. (65)

Moreover, *C1orf21* has been also reported in another GWAS study, conducted in UK. In this investigation a Genome Wide Association Study has been carried out for arterial stiffness index (ASI), and an increase in its expression seems to be associated with an elevated systolic blood pressure (rs1930290, $p = 1{,}1 \times 10^{\wedge -8}$). (66).

Another interesting feature of C1orf21 is that it appears to be stably expressed in skeletal muscle, which may be expected for a modifier of DMD pathology. (65)

The combination of these results leads us to think that the protein product takes a role in development regulation of dystrophic muscle, and therefore in inflammation, regeneration, and fibrosis. Given its putative role in cell cycle regulation, *C1orf21* may be implicated in the proliferation of fibroadipogenic elements that lead to fibro-fatty substitution in muscle, or conversely in the activation of tissue repair by satellite cell activation, or both. The dominant model emerging from our Kaplan-Meier analysis suggests that a regulatory haplotype may enhance or reduce gene expression. Further *in silico* and *in vitro* mechanistic studies are necessary in order to better explain the association, and in particular to characterize the protein or transcript as a potential "druggable" target. In this perspective, it is paramount to identify if the up- or down-regulation of the gene are beneficial or detrimental to skeletal muscle in DMD.

In summary, while these results are yet only preliminary, we have identified a strong candidate for a novel DMD modifier, with a possible large effect on disease progression and LoA.

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